DISSERTATION

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Oral examination:

Analysis of the role of estrogen receptor α in cerebral stroke

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Contents

Zusammenfassung

Das Hormon 17β-oestradiol und sein Rezeptor Östrogen Rezeptor α (ERα) zeigten neuroprotektive Eigenschaften in Tiermodellen für Schlaganfall in Nagetieren. Im Gegensatz dazu legten klinische Studien nahe, daß Langzeitbehandlungen mit Östrogenen das Risiko für Demenz und Schlaganfälle erhöht. Diese Gegensätze zeigen, daß ein besseres Verständnis von E_2 und ERα-vermittelten Wirkungen beim Schlaganfall notwendig ist.

Um die Rolle des ERα im Schlaganfall zu untersuchen, wurden mit Hilfe des Cre-loxP Systems drei zelltypspezifische ERα knock out Mausstämme hergestellt: ein neuronenspezifischer ERα Mutanten-Mausstamm (CaMKIICre/$ER^{fl/fl}$), ein mikroglialer ERα Mutanten-Mausstamm (LysMCre/$ER^{fl/fl}$) sowie ein endothelspezifischer ERα Mutanten-Mausstamm (Tie2CreER^{T2}/$ER^{fl/fl}$). Diese Mausstämme wurden auf ihre gewebsspezifische Inaktivierung des ERα hin untersucht. In den CaMKIICre/$ER^{fl/fl}$-Mäusen fand eine vollständige Inaktivierung des ERα statt. In den LysMCre/$ER^{fl/fl}$-Mäusen war in 92% aller mikroglialen Zellen ERα inaktiviert, wohingegen in den Tie2CreER^{T2}/$ER^{fl/fl}$-Mäusen nur eine unvollständige Inaktivierung des ERα stattfand. Aufgrund dieses Ergebnisses wurden die Tie2CreER^{T2}/$ER^{fl/fl}$-Mäuse von den weiteren Experimenten ausgeschlossen.

Um nun die Rolle vom ERα in Neuronen und mikroglialen Zellen im Schlaganfall zu untersuchen, wurden Experimente mit einem Modell für Schlaganfall, der „middle cerebral artery occlusion" (MCAO), durchgeführt. Nach Analyse der Schlaganfallvolumina nach einer MCAO in CaMKIICre/$ER^{fl/fl}$-Mäusen und LysMCre/$ER^{fl/fl}$-Mäusen, zeigte sich, daß der neuronale ERα und nicht der mikrogliale ERα für die Vermittlung der neuroprotektiven Wirkung von E_2 im Schlaganfall verantwortlich ist. Außerdem wurde gezeigt, daß E_2 nicht nur neuroprotektive Eigenschaften

in weiblichen Tieren, sondern auch in männlichen Tieren besitzt, und daß diese Eigenschaften in beiden Geschlechtern durch den neuronalen ERα vermittelt werden.

Um die molekularen Mechanismen, welche durch den ERα vermittelten neuroprotektiven Effekt beeinflußt werden, besser verstehen zu können, wurde die Genexpreesion in weiblichen CaMKIICre/ER$^{fl/fl}$-Mäusen untersucht. Es wurde in dieser Arbeit gezeigt, daß die Transkription von ERα im Schlaganfall verstärkt stattfindet, wohingegen die Transkripion von Bcl-2, Cocaine- and Amphetamine-regulated transcript (CART), Cyclooxygenase 2 (COX-2), Prostaglandin E2 EP1 Rezeptor (EP1) und Prostaglandin E2 EP2 Rezeptor (EP2) unverändert war. Die Transkription des Brain derived neurotrophic Factor (BDNF) war im Schlaganfall nach E_2 Behandlung erhöht. Diese verstärkte Transkription des BDNF-Gens war unabhängig vom neuronalen ERα, da auch in den neuronalen ERα knock out Mäusen die Transkription erhöht war.

Zusammengefasst wurde in dieser Arbeit gezeigt, daß der neuronale ERα und nicht der mikrogliale ERα eine essentielle Rolle in der E_2 vermittelten Neuroprotektion im Schlaganfall spielt.

Summary

The hormone 17β-estradiol (E_2) and its receptor estrogen receptor α (ERα) have neuroprotective effects in animal models of stroke in rodents. In contrast, clinical studies revealed, that long term treatment with estrogens lead to an increased risk of dementia and stroke. These controversy shows, that there is a need for a better understanding of E_2 and ERα action in stroke.

To investigate the role of ERα in stroke, three cell type specific ERα knock out mouse-strains were generated using the Cre-loxP system: a neuronal specific ERα knock out mouse strain (CaMKIICre/$ER^{fl/fl}$), a microglial specific ERα knock out mouse strain (LysMCre/$ER^{fl/fl}$) and an endothelial specific ERα knock mouse strain (Tie2CreER^{T2}/$ER^{fl/fl}$). These mouse-strains were analysed for tissue specific deletion of ERα. Deletion of ERα in neurons of CaMKIICre/$ER^{fl/fl}$-mice was complete, in LysMCre/$ER^{fl/fl}$-mice ERα was deleted in 92% of the microglial cells whereas the deletion of ERα was incomplete in endothelial cells of the vascular system in the Tie2CreER^{T2}/$ER^{fl/fl}$-mice. Due to these results the Tie2CreER^{T2}/$ER^{fl/fl}$ mouse-strain was excluded for further experiments.

To investigate the role of ERα in neurons and in microglial cells in stroke, experiments using a model of middle cerebral artery occlusion (MCAO) were performed. Analysing the stroke volume after performing a MCAO in CaMKIICre/$ER^{fl/fl}$-mice and in LysMCre/$ER^{fl/fl}$-mice revealed, that it is neuronal ERα and not microglial ERα which mediates the neuroprotective effects of E_2 in stroke. Furthermore it was shown, that E_2 has neuroprotective effects in female as well as in male mice, and that in both sexes the neuroprotecive effect of E_2 is mediated via neuronal ERα.

For a better understanding of the molecular mechanisms underlying these neuroprotective effects mediated by neuronal ERα, the expression of several genes in female CaMKIICre/$ER^{fl/fl}$-mice was investigated. It was shown in this work, that ERα is upregulated in stroke, whereas Bcl-2,

cocaine- and amphetamine-regulated transcript (CART), cyclooxygenase 2 (COX-2), prostaglandin E2 EP1 receptor (EP1) and prostaglandin E2 EP2 receptor (EP2) transcription was unchanged. Brain derived neurotrophic factor (BDNF) was upregulated upon E_2 treatment in stroke. The upregulation of BDNF was independent from neuronal ERα since its transcription was elevated in the neuronal ERα knock out mice as well.

Taken together, it was demonstrated in this work, that neuronal ERα and not microglial ERα plays a major role in E_2 mediated neuroprotection in stroke.

1. Introduction

Estrogens and their receptors are involved in many regulatory and protective signalling pathways. Mostly known to play an essential role in female reproductive and sexual differentiation processes, evidences accumulated that estrogens play an important role in the vascular and in the central nervous system. It has been described that estrogens and their receptors have neurotrophic and neuroprotective effects in diseases like Alzheimer and stroke. Several studies suggested that the neuroprotective effects of estradiol in stroke are mediated via its receptors estrogen receptor α and estrogen receptor β but it remained unknown in which cells this protective effect occurs. In this work it was shown that these neuroprotective effects in stroke are mediated via neuronal and not microglial estrogen receptor α by using-tissue specific estrogen receptor α knock out mice as a model of ischemic stroke. It was also shown that these estrogen receptor α-mediated neuroprotective effects of estradiol are present in female mice as well as in male mice.

1.1 Estradiol

17β-Estradiol (E_2) is mainly synthesized in the granulosa cells of the ovaries and plays several important roles in reproductive organs and in the central nervous system:

- E_2 is essential for reproduction in mammals, it plays a pivotal role in pubertal development, regulation of the estrous cycle and establishment and maintenance of pregnancy and lactation (Hewitt et al., 2005).
- Cognitive functions like verbal fluency, performance on spatial tasks, verbal memory tests and fine motor skills are influenced by E_2,

showing that E_2 prevents cognitive decline (Merchenthaler et al., 2003).

- Thermo-regulation is affected by E_2. The low E_2 level in postmenopausal women causes hot flushes and night sweating (Schmidt et al., 2006).
- E_2 also acts as a general neurotrophic factor that stabilizes neuronal function and supports viability. The role of E_2 in neurodegenerative diseases, e.g. Alzheimer´s disease and Parkinson´s disease reflects its function as a neurotrophic factor in the central nervous system. E_2 reduces the risk of the onset and delays the progression of such diseases (Behl, 2002).
- In the central nervous system, E_2 was shown to have protective effects against brain injury and neurodegeneration (Merchenthaler et al., 2003). For example, *in vitro* and *in vivo* studies have described neuroprotective actions of estrogens in serum deprivation, glutamate-induced excitotoxicity (McEwen and Alves, 1999) and in a variety of models of acute cerebral ischemia where it represses apoptosis in ischemic incidences of the brain (Rau et al., 2003).

In other organ systems it has been shown that E_2 has beneficial effects in the prevention of cardiovascular diseases resulting from atherosclerosis. E_2 causes rapid vascular dilatation and significantly inhibits vascular smooth muscle cell proliferation after injury (Pare et al., 2002). It has also long-term effects on gene expression in vascular cells and lipoprotein level-changes. Additionally it plays an important role in bone formation (Sims et al., 2003).

Two mechanisms have been suggested to mediate estrogen effects in the brain and in the vascular system:

- Classical genomic actions which involve estrogen receptor- (ER) mediated gene transcription. The upregulation of neurotrophic factors like brain derived neurotrophic factor (BDNF), insuline-like growth factor-1 (IGF-1), nerve growth factor (NGF) and other neutrophins were described as possible candidates for mediating

neuroprotection (Garcia-Segura et al., 2001; Wise et al., 2001; Zhao et al., 2004). E_2 also influences apoptosis by downregulation of proapoptotic genes like Bad and Cox-2, and upregulation of antiapoptotic genes like Bcl-2, Bcl-$_{XL}$ (Dubal et al., 1999; Nilsen and Diaz Brinton, 2003; Pike, 1999).

- nongenomic actions of ligand bound ER by affecting MAPK- (Szego et al., 2006) and/or PI3K-pathways (Choi et al., 2004). Non-genomic neuroprotective actions of E_2 are described in microglial cells and endothelial cells of the vascular system. In microglial cells, E_2 is thought to prevent via ERα the translocation of the NFκ-B subunit p65 and thereby repressing the transcription of proinflammatory cytokines (Ghisletti et al., 2005), whereas in endothelial cells E_2 prevents the adhesion and migration of leukocytes to and through the endothelial cell layer via stimulation of the PI3-K pathway, and upregulation of the eNOS activity (Simoncini et al., 2000).

Additionally, receptor-independent actions of estrogens, like antioxidant characteristics (Behl et al., 1997) have been suggested.

In clinical treatment, estrogens became an object of controversial discussion regarding its role in the hormone replacement therapy (HRT). Estrogens are the only active substances to treat symptoms like hot flushes, night sweats and sleep disturbances caused by menopause and they have beneficial effects in the treatment of osteoporosis. Beside these beneficial effects of estrogens in HRT, it turned out that long term treatment with estrogens leads to a higher risk of endometrial cancer and ovarian cancer. Furthermore long term HRT leads to an elevated risk for dementia and stroke caused by increased blood clotting (Schmidt et al., 2006). The circumstance that estrogens on the one hand lead to a higher risk of stroke insults in the case of long term treatment, but on the other hand have shown to be beneficial in experimental models of stroke, points out the need for a better understanding of estrogen actions in stroke.

1.2 Estradiol and stroke

Stroke is a global epidemic and an important cause of morbidity and mortality. It ranks next to cardiovascular disease and cancer as a cause of death. The estimated direct and indirect cost of stroke for 2007 in the US is $62.7 billion (Rosamond et al., 2006). Stroke is also the leading cause of adult disability, because 76% of people survive their stroke. Of these survivors, 50% have a hemiparesis, 26% are dependent in activities of daily living, and 26% are forced into a nursing home. Possible signs and symptoms of stroke are unilateral weakness or paralysis, a sagging of one side of the face, double or blurred vision, vertigo, numbness or tingling, and language disturbances (Zerwic et al., 2002). There are two major classifications of stroke:

- 13% are classified as hemorrhagic strokes, which are caused by the rupture of a cerebral blood vessel and bleeding in the surrounding tissue. Most common causes for aneurysms are hypertension and atherosclerosis.
- 87% of all strokes are ischemic strokes. An ischemic stroke results from the complete occlusion of an artery.

The characteristic of ischemic stroke is evolving damage, in which ischemic cell death or cell stress responses progress after the initial ischemic insult. The region of the ischemic penumbra, a brain region adjacent to the earliest region of ischemic cell death will progress to infarction over time unless untreated and is followed by secondary mechanisms of ischemic cell death such as inflammation and oxidative injury. Epidemiological studies show men and postmenopausal women are at a higher risk for stroke than premenopausal women. In animal models of stroke, estrogens impair the progression of ischemic cell death and lead to smaller infarct areas in estradiol treated animals compared to untreated animals (Dubal et al., 1999). Nevertheless, so far these studies have failed to show in which celltype the beneficial effects of estradiol and its

receptors take place. A better understanding of the estradiol mediated neuroprotective effects on a cellular as well as on a molecular level are a necessary prerequisite to devise clinical applications that seize E_2's positive effects and circumvent unwanted side effects.

1.3 Estrogen receptors

There are two known estrogen receptors in mice, estrogen receptor α (ERα) and estrogen recptor β (ERβ). The two receptors are distinct proteins encoded by separate genes (Esr1 and Esr2) located on different chromosomes. Both are ligand-dependent transcription factors and members of the nuclear hormone receptor superfamily (ERα classified as NR3A1 and ERβ classified as NR3A2). They share a high homology in some domains, like the DNA binding domain (96% homology), but differ in the ligand binding domain (58% homology) and the N-terminal transactivation domain.

ERs and estrogens as their ligands act in an apparently simple pathway. Estrogens, like all steroid hormones, are small lipophilic molecules which diffuse from the blood through the cell membrane into the cytosol and the nucleus. Without a ligand, the majority of the ERs are blocked in the nucleus by a complex of heat shock proteins. After binding a ligand to the ER, the heat shock proteins dissociate and the receptor gets into an activated form. Activated ERs dimerize and bind to specific DNA sequences (Beato et al., 1995). These sequences are called estrogen response elements (EREs) and consist of two hexanucleotides which contain an inverted repeat sequence, separated by a spacer of three nucleotides. Binding of the ERs to EREs leads to changes of gene expression in the cell. Another way to influence gene expression by ligand bound ER are protein-protein interactions with transcription factors as e.g. the AP-1 family and therefore changing transcription of genes without binding directly to DNA (Gottlicher et al., 1998).

Beside these "classical" ER mechanisms, there are also so called "nongenomic" or "rapid" estrogen actions (Hall et al., 2001). These nongenomic actions occur after a few minutes of E_2 treatment and they influence pathways like the MAP-kinase (MAPK)- and phosphatidylinositol-3'-kinase (PI3K) pathways (Simoncini et al., 2000). However, the molecular mechanisms of nongenomic effects remain controversial (Pedram et al., 2006).

Transcription of the ERα gene in the mouse results in a single transcript of 6.3 kb, transcribed from 9 Exons (Fig. 1). This transcript encodes a protein of 599 amino acids with a molecular mass of 66 kDa.

In contrast the ERβ protein is composed by 530 amino acids with a molecular mass of 60 kDa. The majority of this difference in size between the two ERs is due to a significantly shorter N'-terminus in the ERβ protein.

The ERα and ERβ proteins are composed of six functional domains (Fig. 1), labelled A-F. The N'-terminal A/B domain contains a ligand-independent transactivation domain (AF-1) and is encoded by exon 2. The C domain is the DNA-binding domain (DBD) encoded by exon 3 and 4. It is characterized by two zinc fingers, which form the DNA-binding domain responsible for binding to EREs. The nuclear localisation signal in the D domain is encoded by exon 5, followed by the E and F domain which possess the ligand binding domain (LBD) and the ligand-dependent transactivation domain (AF-2). Both domains, the DBD and the LBD are necessary for dimerization of the receptor.

1.4 Estrogen receptor knock out mice

The first available ERα knock out mouse (here called ERαKOneo) was generated by Korach and co-workers in 1993 by cloning a neomycin-resistance gene (neo) into exon 2 of the Esr1 gene, thereby disrupting it

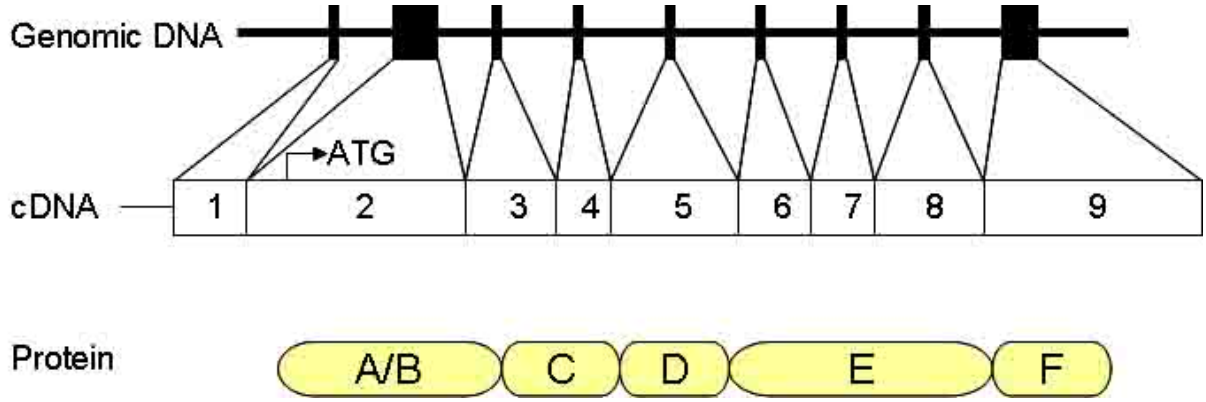

Fig. 1 Scheme of the Esr1 gene and its translation into the ERα protein
The gene consists of 9 Exons which are translated into a protein with five domains.

(Lubahn et al., 1993). Later analysis revealed an incomplete deletion of the ERα protein (Pendaries et al., 2002). The ERαKOneo mouse expresses a truncated form of the ERα protein with a molecular weight of 61 kDa at lower levels than the wildtype protein. The ERβ knock out mouse was established in 1998. The Esr2 gene was inactivated by insertion of a neo-cassette in exon 3 (Krege et al., 1998).

A second ERαKO (here called ERαKO) and a second ERβKO mouse line (here called ERβKO) were reported in 2000, both lacking exon 3, which results in a complete loss of the ERα respectively the ERβ (Dupont et al., 2000). The phenotypes of the ERαKO and the ERβKO are quite different, reflecting that both proteins have different implications (Hewitt and Korach, 2003).

ERαKO suffer from elevated luteinizing hormone (LH), estrogen, testosterone and low prolactin levels. In the ERβKO no changes of these hormone levels are observed. Both knock outs have reduced ovulations in superovulation trials. The follicles of ERαKO are immature and hemorrhagic cystic follicles begin to develop at the beginning of puberty as a result of chronic elevated LH. The ERβKO mice show impaired follicle development, which leads to subfertility. Ovulation can be induced in ERαKO by treating young mice with exogenous gonadotropins before LH

rises, showing that ERα is not important for follicle maturation and rupture (Couse et al., 1999). However, ERα is indispensable for the regulation of ovulation by the HPG axis (Wintermantel et al., 2006).
It was demonstrated that estradiol is still able to inhibit the vascular injury response in the ERαKOneo and the ERβKO, but it is abolished in the ERαKO (Pendaries et al., 2002).

1.5 Conditional mutagenesis

The ERαKO model is a powerful tool for the understanding of ERα actions in mice, but is limited for the explanation of particular endocrine circuits and cell type specific events influenced or regulated by ERα. Since the receptor is ablated in all tissues, phenotypes can also occur due to side effects from the integration of several disregulated circuits as well as due to disturbed developmental processes.

1.5.1 The Cre-loxP-system

The Cre-loxP system is a genetic technology which gives the possibility for conditional mutagenesis of a gene of interest (Nagy, 2000). This means, that the gene of interest gets inactivated not only under certain circumstances but also in defined tissues of the mouse. This technology allows the investigation of the role of an organ in a complex endocrine dysfunction as well as the role of a gene in a certain organ or celltype without distortion caused by systemic influences.
The Cre-loxP-system was first described in bacteriophages and consists of two components (Gu et al., 1994): a sequence-specific recombinase (Cre, a 36 kDa protein) and a DNA sequence flanked by loxP sites (34 bp DNA elements, which are recognized by the Cre-recombinase, Fig. 2A). The Cre-recombinase (Cre) catalyses recombination of two loxP-sites (Fig.

2B). If both loxP-sites have the same orientation, the loxP-flanked DNA will be excised and thereby eliminated. There are no metabolic compounds or cofactors necessary to catalyze this reaction. This system is also working in *E.coli*, yeast, plants and more complex organisms.

In mice the Cre-loxP system has to fulfil two requirements to give rise to a conditional mutagenesis:

- Tissue specific Cre expression

  Tissue specific Cre expression is achieved by putting the Cre gene under the control of an appropriate promoter. This means, that the tissue-specifity of the Cre-mediated recombination is given by the promoter controlling the expression of the Cre. The transgene, consisting of the chosen promoter and the Cre gene, should mimic the expression pattern of the endogenous gene. It has been shown that, using small vectors (<10kb), which contain only few elements of a promoter, can result in mosaicism or broader expression of the transgene than the endogenous expression pattern (Tronche et al., 1999). Bacterial artificial chromosomes (BACs) can accommodate more than 150 kb of a transgene, thereby containing most of the 5'- and 3'-promotor elements of the endogenous promoter to guarantee the expression pattern of the tissue specific promoter of interest. Additionally it was shown that large sized transgenes are expressed independent of the integration site in the genome and that expression is only dependent on the copy number of the transgene (Schedl et al., 1993).

- Gene inactivation by using loxP-sites

  Flanking an essential Exon of a gene by loxP-sites and recombining these sites by the Cre, leads to the loss of the Exon sequence and thereby to the loss of the capability to translate a functional protein. In case of the ERαloxP mouse ($ER\alpha^{fl/fl}$-mouse), the loxP-sites are integrated in the introns 5' and 3' of Exon 3 of the Esr1 gene by gene targeting (Wintermantel et al., 2006). Placing the loxP sequences into Introns should not have any effects on the

expression of the gene. Exon 3 of the ERα, like Exon 3 of all steroid hormone receptors, is essential for the translation of the RNA to a functional protein. Therefore excising Exon 3 of the Esr1 gene, by using the Cre-loxP system, leads to a complete loss of the protein.

In addition to allowing cell type-specific recombination, it is also possible to set a timepoint for the mutagenesis (Feil et al., 1997) by the introduction of a fusion protein consisting of the Cre fused to a mutated ER ligand binding domain ($CreER^{T2}$). The mutated ligand binding domain of the ER can only bind tamoxifen and is not able to bind other estrogens anymore. The $CreER^{T2}$ without tamoxifen as a ligand, is inactivated by heatshock proteins in the cytoplasm. After tamoxifen treatment, the heatshock proteins dissociate from the $CreER^{T2}$ and the recombinase is translocated to the nucleus where it catalyses the recombination (Fig. 2C). Therefore the $CreER^{T2}$ allows inducible cell type specific mutagenesis.

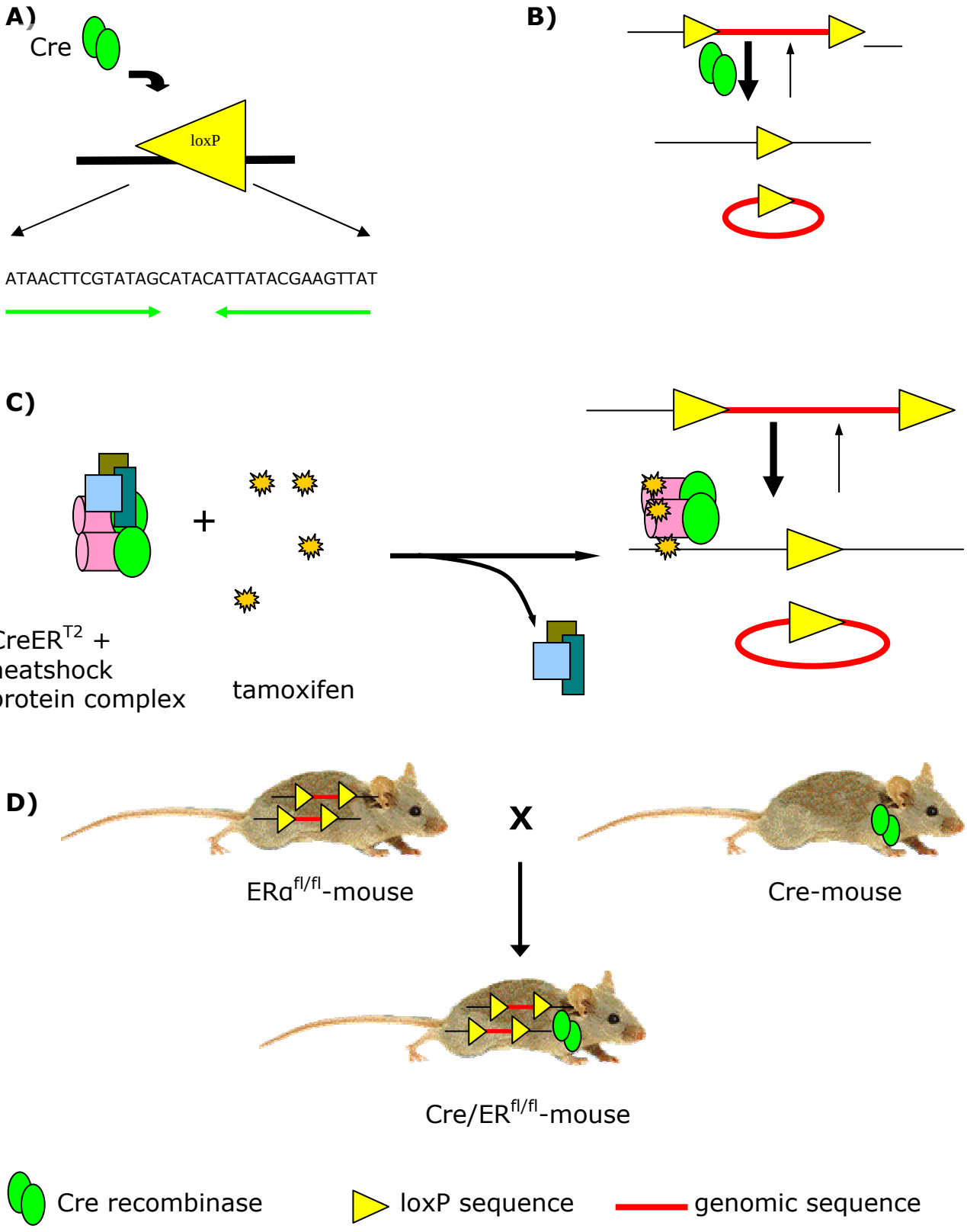

Fig.2 Conditional mutagenesis using the Cre-loxP-system
A) the Cre recognizes the loxP site and binds to it. B) The Cre catalyses the recombination of two loxP sites. If both loxP sites have the same orientation, the flanked sequence will be excised and thereby deleted. C) Heatshock proteins dissociate upon tamoxifen binding to the CreER^{T2}. After translocation of the CreER^{T2} into the nucleus, the recombination of the floxed alleles take place. D) Breeding mice carrying a loxP flanked (floxed) locus with mice, expressing the Cre in a celltype-specific manner, leads to mice carrying both transgenic alterations. The recombination takes place and results in a celltype-specific deletion of the floxed gene.

1.6 Aim of this thesis

In stroke the ERαKO shows no estradiol mediated neuroprotective effect. In the ERβKO estradiol treatment is still as efficient as in wildtype mice related to neuroprotection (Dubal et al., 2001).Thus it is proven that the protective effects of E_2 in stroke are mediated via ERα and not ERβ. However, the question still remains in which celltype ERα action takes place. To investigate this issue, we focused on three cell types, which are thought to play an important role while a stroke occurs.

1.6.1 Endothelial cells of the vascular system:

To elucidate the role of ERα in endothelial cells while a stroke occurs, we generated and analysed a transgenic mouse expressing the $CreER^{T2}$ under control of the Tie2-promoter ($Tie2CreER^{T2}$). Breeding these mice to $ER\alpha^{fl/fl}$-mice should result in an endothelial specific ERα knock out mouse upon tamoxifen treatment ($Tie2/ER^{fl/fl}$).

1.6.2 Microglial cells:

Microglial cells as "macrophages of the brain" are thought to play an important role in the inflammatory response and apoptosis in stroke. Breeding mice, expressing the Cre under control of the lysozyme M promoter (Clausen et al., 1999) to $ER\alpha^{fl/fl}$-mice, results in a ERα knock out specific in the monocytic lineage ($Lys/ER^{fl/fl}$).

1.6.3 Neurons of the forebrain:

To achieve a specific ERα knock out in neurons of the forebrain, mice transgenic for a Cre under the control of the CaMKIIα promoter (Casanova et al., 2001) were bred with $ER\alpha^{fl/fl}$-mice to obtain an ERα knock out in the neurons of the forebrain ($CaMKIICre/ER^{fl/fl}$) (Wintermantel et al., 2006).

The aim of this work is to identify the cell type in which the beneficial effects of E_2 are mediated via the ERα in stroke. Additionally, potential pathways influenced by ERα are investigated and elucidated.

2. Materials and methods

2.1 Chemicals

Chemicals were obtained from the following companies:

- Fluka, Neu-Ulm
- Merck, Darmstadt
- Carl Roth GmbH, Karlsruhe
- Sigma-Aldrich Chemie GmbH, Steinheim

2.2 Enzymes

Taq-DNA-polymerase	Roche Molecular Biochemicals, Mannheim
Proteinase K	Carl Roth GmbH, Karlsruhe
RNAse A	Qiagen, Hilden
SuperScript II RT	Qiagen, Hilden

2.3 Primers

Primers were obtained from MWG-Biotech AG, München.

Primers to detect the Tie2CreER^{T2}-transgene:
MWG 503: 5'-GAAGTCGCAAAGTTGTGAGTTG-3'
MWG 504: 5'-TGGCTTGCAGGTACAGGAG-3'
MWG 505: 5'-GAGAATGGCGAGAAGTCACTG-3'

Primers to detect the LysMCre-transgene:
Lys-forward: 5'-GCTTTCTCTAGTCAGCCAGCAG-3'

Cre-reverse: 5’-AGCATTGGAGTCAGAAGGGCGT-3’

Primers to detect the CaMKIIαCre-transgene:
CaMKII1: 5’-GGTTCTCCGTTTGCACTCAGGA-3’
CaMKII2: 5’-CCTGTTGTTCAGCTTGCACCAG-3’
CaMKII5: 5’-CTGCATGCACGGGACAGCTCT-3’

Primers to detect the ERα-loxP allele:
MWG 539: 5’-TAGGCTTTGTCTCGCTTTCC-3’
MWG 540: 5’-CCCTGGCAAGATAAGACAGC-3’
MWG 541: 5’-AGGAGAATGAGGTGGCACAG-3’

Primers to detect the RAGE-eGFP allele:
MWG 531: 5’-CTGGGTGCTGGTTCTTGCTCTA-3’
MWG 532: 5’-GTTCTGACCACCAGCTACAGCT-3’
MWG 533: 5’-GGCATGGCGGACTTGAAGAAGT-3’

2.4 Buffers and standard methods

2.4.1 Production of genomic DNA-lysates from mouse-tails for genotyping PCRs:

NID-buffer

50 mM KCL
10 mM Tris pH 8.3
2 mM $MgCl_2$
0.1 mg/ml gelatine
0.45% NP 40
0.45% Tween 20
1 mg/ml Proteinase K

Mouse tails were digested in 200 µl of NID-buffer overnight at 56°C. After inactivation of the proteinase K at 95°C for 20 min, 1 µl of the lysate was used for the PCR reaction.

2.4.2 Analytical PCR for genotyping

PCR reaction mix

1 µl genomic DNA-lysate, preparation see 2.4.1
2.5 µl 10x PCR buffer
1 µl dNTP-mix (5 mM dATP, dTTP, dGTP, dCTP)
6 pmol each primer
0.5 U Taq-DNA-polymerase
Add H_2O to a volume of 25 µl

PCR-programs:

ERα-loxP PCR

95°C – 5’

For 35 times
95°C – 30’’
61°C – 30’’
72°C – 1’

72°C – 7’

CaMKIIαCre-, LysMCre-, RAGE/^{eGFP}-loxP-PCR

95°C – 5’

For 35 times
95°C – 30’’
63°C – 1’
72°C – 1’

72°C – 7’

Tie2CreER^{T2}-PCR

95°C – 5’

For 35 times
95°C – 30’’
58°C – 30’’
72°C – 1’

72°C – 7’

PCR results were analysed using 2% agarose gel-electrophoresis. DNA was visualized with UV-light using 0.5 µg/ml ethidiumbromide.

2.4.3 Buffer for agarose-gelelectrophoresis

50x Tris-acetatebuffer (TAE)

2 M Tris
250 mM Na-acetate

50 mM EDTA pH 8

Acetic acid is used to adjust pH to 7.8

6x sample buffer

0.25% bromphenolblue

0.25% xylenecyanol FF

15% Ficoll 400

DNA-sizemarker:

"Smart Ladder", Stratagene. 5 µl per lane.

2.4.4 PBST for immunohistochemistry

PBS:

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

pH is adjusted to 7.2 using HCl

PBST for immunohistochemistry on paraffinsections:

Tween 20 added to PBS to a final concentration of 0.02%

PBST for immunohistochemistry on vibratomsections, frozen sections and for immunocytochemistry on microglial cells:

Triton-X-100 added to PBS to a final concentration of 0.2%

2.5 Mouse strain background

All mouse strains were crossed to a C57/Bl6 background. C57/Bl6-mice were obtained from Charles River. Crossing the mice for four generations to a C57/Bl6 background guarantees a nearly complete C57/Bl6 background.

2.6 Tamoxifen solution and induction protocol

0.5 mg tamoxifen were solved in 0.5 ml 100% EtOH and 4.5 ml sun flower seed oil (Sigma). The solution was mixed at 4°C overnight.
Mice were injected intra peritoneal with 100 µl tamoxifen solution (= 1mg tamoxifen) each day for five consecutive days. Fowolling another nine days for recovery and that recombination takes place, mice were sacrificed for further analysis.

2.7 Preparation of sections

Frozen sections were prepared using a cryostat (Leica CM 3050). 6 µm paraffin sections were prepared using a microtome (Leica). 40 µm free floating sections were prepared using a vibratome (Microm).

2.7.1 Preparation of frozen sections and visualization of eGFP positive cells of Tie2CreER^{T2}/RAGE$^{eGFP/+}$-mice

Organs were isolated and immediately frozen in liquid nitrogen cooled isopentane. 10 µm Frozen sections were briefly dried and embedded in Vectashield mounting medium for fluorescence (Vector laboratories). EGFP

positive cells were visualized by fluorescence microscopy using filter set 24 (Zeiss).

2.7.2 Preparation of frozen sections for the analysis of the stroke volume and TUNEL-histochemistry

The brains of mice which underwent a MCAO were isolated and immediately frozen on dry ice. 20 µm coronal serial sections every 400 µm of the forebrain were performed and collected on Polysin™ slides (Menzel Gläser). Sections were then used to perform a silver stain or TUNEL-histochemistry.

2.7.3 Preparation of paraffin sections

Organs were isolated and fixed in 4% para-formaldehyde/PBS (4% PFA) at 4°C overnight. Organs were then washed twice for 30 min with PBS at room temperature and dehydrated using an ethanol-gradient: 2 x 30 min 70% ethanol, 1 x 30 min 85% ethanol, 1 x 30 min 95% ethanol, 3 x 100% ethanol, 1 x 30 min xylene, xylene overnight, 1 x 30 min xylene. Organs were then incubated in 60°C paraffin, 3 x 60 min. Finally, the organs were imbedded in 60°C paraffin and cooled down. 6 µm Paraffin sections of the embedded organs were prepared using a Leica microtome. The sections were collected on SuperFrost-slides and incubated at 56°C overnight to stick the sections on the slide. The slides were stored at room temperature.

2.7.4 Peparation of free floating sections

Brains were isolated and fixed for 48 h in 4% PFA at 4°C. 40 µm coronal sections of the fixed brains were prepared using a vibrotome. Free floating sections were stored in 0.5% PFA for up to six months.

2.8 Immunohistochemistry

Antibodies:
Polyclonal anti-ERα MC-20, Santa Cruz sc-542, diluted 1:2000 in 5% normal swine serum (DAKO)/PBST (5% NSS).

Biotinylated anti rabbit antibody, Vector laboratories Burlingame USA, diluted 1:400 in PBST.

Detection system:

ABC-peroxidase system, Vectastain, Vector laboratories used with DAB (Sigma) as a substrat. DAB gets converted by the ABC-peroxidase system into a brown precipitate.
Hematoxylin counterstain was performed using Hematoxylin QS, Vector laboratories

2.8.1 ERα detection on paraffin sections

To remove the paraffin, the slides were incubated 3 x 5 min in xylene. Afterwards, sections were rehydrated using an ethanol gradient: 2 x 5 min 100% ethanol, 1 x 5 min 95% ethanol, 1 x 5 min 85% ethanol, 1 x 5 min 70% ethanol, 2 x 5 min PBS. Endogenous peroxidase activity was blocked with 50% MeOH/PBS 3% H_2O_2. Slides were washed twice for 5 min in PBS

and boiled in Antigen-Retrieval buffer (DCS, Hamburg), first 2 min 800 W than 8 min 360 W, to expose the antigene. After cooling and washing with PBS the slides, sections were incubated for 10 min with 5% NSS blocking solution. Slides were then incubated overnight with primary antibody at 4°C overnight. Following primary antibody incubation, slides were washed 2 x 5 min with PBS and incubated for 30 min with secondary antibody. Afterwards the slides were washed again 2 x 5 min with PBS and incubated for 30 min with ABC-peroxidase system. Detection was performed using DAB substrate resulting in a brown precipitate. Counterstain was performed by incubating the sections 1 min with Hematoxylin QS.
The sections were dehydrated (see below) and after incubation in xylene 3 x 5 min embedded in Eukitt.

2.8.2 ERα detection on vibratome sections

Sections were collected in 24-well plates and washed with PBS. Endogenous peroxidase activity was blocked with 50% MeOH/PBS 3% H_2O_2 for 15 min. Afterwards sections were washed 3 x 10 min with PBST. Blocking of unspecific binding sites was achieved by incubating the sections with 5% NSS for 30 min. Sections were incubated with the primary antibody at 4°C overnight. Following washing 3 x 10 min with PBST, sections were incubated with secondary antibody for 30 min. After washing 3 x 10 min with PBST, the sections were incubated for 30 min with ABC-peroxidase system and washed 2 x 10 min with PBS. Detection was performed using DAB as a substrate.
After staining with DAB, the sections were dried and incubated with xylene. The sections were then mounted with Eukitt.

2.9 TUNEL-histochemistry

TUNEL-staining was performed using DeadEnd Fluorometric TUNEL System (Promega Corporation, Wisconsin USA). Buffers and reaction mix were prepared according to the technical bulletin.
20 µm frozen sections of brains of mice, which underwent a MCAO, were thawed and fixed for 10 min with 4% PFA. Sections were washed 2 x 5 min with PBS, following incubation with PBST for 30 min. After washing the sections 2 x 5 min with PBS, sections were incubated with TDT-reaction-buffer. Sections were then incubated with TUNEL-reaction mix at 40°C for 2 h. Afterwards sections were washed 3 x 5 min with PBS and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Slides were stored at 4°C until fluorescence microscopy analysis.

2.10 Microglial cell culture and immunocytochemistry

2.10.1 Isolation of microglial cells from mouse brains

Microglial cells were isolated from newborn mice (P1) as described (Burudi et al., 1999).

2.10.1.1 Buffers and media

- Poly-L-lysine, ready-to-use 0.01% solution (Sigma Cat.: P-4832)
- Dnase from bovine pancreas grade II, (Roche Diagnostics Cat. 104159)
- Trypsine 2.5% solution (10x) (Invitrogen Cat. 25090-010)
- HEPES 1M solution (Sigma Cat.: H 0887)
- HBSS 1 M solution (Sigma Cat.: H 1641)

- Trypsine-EDTA (low): typsine 0.05%, EDTA 0.02%, in HBSS without Ca^{2+}/Mg^{2+} (Invitrogen, Cat.: 25300-054)
- Trypsine-EDTA (high): trypsine 0.025%, EDTA 0.04%, in HBSS without Ca^{2+}/Mg^{2+} (Invitrogen, Cat.: 25200-056)
- DMEM with 4500 mg glucose, L-glutamine and sodium bicarbonate (Sigma Cat.: D 5796)
- Penicillin-Streptomycin (Invitrogen, Cat.: 15140-122)
- Glutamine (Invitrogen, Cat.: 25030-024)
- Gentamycin (Invitrogen, Cat.: 15750-045)
- Fetal calf serum (GIBCO), 30 min inactivation at 56°C
- PLL 0.01%: 0.01g PLL dissolved in PBS. The solution was filtered with 0.45 µm Millipore-filter and stored at 4°C.
- HBSS 1x: 1x HBSS/100 mM HEPES pH 7 stored at 4°C.
- DNase solution: 0.05% DNase/HBSS pH 6.8 stored at -20°C.
- Trypsine solution: 1% Trypsin/DNase 0.5 mg/ml in HBSS pH 7.8 stored at -20°C
- Growth medium (cDMEM): DMEM, 10% FCS, glutamine 1%, Penicillin-Streptomycin 1%

2.10.1.2 Microglial cell culture

75 cm^2 flasks were coated with PLL 0.01%, one flask for three brains. Brains of one day old mice were isolated under sterile conditions. The brains were collected in a cell culture dish containing HBSS and the meninges were removed under a binocular using two forceps. Afterwards the brains were collected in 50 ml Falcon tubes containing 10 ml HBSS/DNase 0.05% and incubated for 3 min at room temperature. Brains were homogenized by pipetting 4-5 times the brains with a 10 ml glass pipette. The lysates were incubated for 20 min at room temperature after adding 1 ml 1% trypsine. In the mean time the PLL was removed from the flasks and 9 ml cDMEM was put into the flasks. After 20 min the Falcon

tubes containing the cell lysate were filled to a volume of 50 ml with cDMEM and centrifuged for 10 min at 180 g. The supernatant was discarded and the precipitate was resuspended with 1 ml cDMEM per three brains. The cell lysate was distributed with 1 ml per flask and incubated at 37°C, 5% CO_2. The medium was changed at day 1, day 2 and day 7.

After 14 days the secondary culture for the selection of the microglial cells was performed. All steps were done at room temperature. All volumes are given for the treatment of one flask.

Flasks were shaken vigorously up to 10 times to detach oligodendrocytes and microglial cells bound at the surface of the cell layer. The medium was discarded and the flasks were rinsed once with 10 ml cDMEM each. Flasks were incubated with 3 ml trypsine-EDTA per flask for 3 min at room temperature. The trypsine-EDTA was discarded and 10 ml of cDMEM and 0.5 ml of DNase were added. The cells were resuspended with a Pasteur pipette. The cell suspension was transferred to 15 ml Falcon tubes and centrifuged for 10 min at 180 g. Meanwhile 2 ml of cDMEM were distributed to Petri dishes (bacterial grade, Sarstedt) and coverslips were put into the dishes. After centrifugation the cell precipitate was resuspended with 4 ml of cDMEM. 1 ml of the resuspended cells was given to each Petri dish and was incubated at 37°C, 5% CO_2 for 20 min. After 20 min incubation, 6 ml cDMEM was added to the cultures and the cells were grown at 37°C, 5% CO_2. Medium was changed once a week. The microglial cells were used after three weeks of isolation for further analysis.

2.10.2 ERα detection and determination of recombination efficiency in microglial cells of LysMCre/ER$^{fl/fl}$-mice

Antibodies:
Polyclonal anti-ERα MC-20, Santa Cruz sc-542, diluted 1:500 in 5% NSS.

Isolectin GS-IB_4 AlexaFluor 488 from Griffonia simplicifolia, Invitrogen, diluted 1:20 in 5% NSS.

Secondary antibody anti-rabbit AlexaFluor 594, Invitrogen, diluted 1:500 in 5% NSS.

Mounting medium:

2.4 g Mowiol (Hoechst) were solved in 6 ml 1% glycerol and incubated for 2 h at room temperature. Afterwards, the solution was mixed with 12 ml 0.2 M Tris-HCl pH 8.5 and incubated at 50°C for 10 min. The prepared Mowiol was stored at -20°C.

Protocol:
Coverslips with attached microglial cells were washed 2 x 5 min with PBS/$MgCl_2$, following fixation with 4% PFA. Afterwards the cells were treated for 10 min with 50 mM NH_4Cl/PBS and permeabilised for 15 min with 0.1% Triton-X-100/PBS. The cells were washed 1 x 5 min with PBS/MgCl and unspecific binding sites were blocked for 20 min with 5% NSS. Incubation with anti-ERα antibody was performed at 4°C overnight. The cells were washed 3 x 5 min then and incubated for 30 min with the secondary antibody. Following second antibody incubation, the cells were washed 3 x 5 min with PBS/$MgCl_2$ and incubation of the cells with the isolectin B_4-antibody was performed at 4°C overnight.Finally the cells were washed 3 x 5 min with PBS/$MgCl_2$ and coverslips were mounted with Mowiol.

ERα positive cells and isolectin B_4 positive cells were counted using fluorescene microscopy. Recombination efficiency was determined by the ratio of ERα positive cells/ isolectin B_4 positive cells. 400 cells were counted. Microglial cells of ERα$^{fl/fl}$-mice were stained as a control.

2.11 Middle cerebral artery occlusion

MCAO was performed in mice as described (Zhang et al., 2005).

8 week old female mice were anesthetized by intraperitoneal injection of 150 µl 2.5% avertin per 10 g body weight and ovariectomized. Female mice as well as male mice received an E_2-pellet 0.025 mg 21 days release (Innovative Research of America, Sarasota, Florida USA) to achieve a constant E_2-plasmalevel of 35 pg/ml (Horsburgh et al., 2002) whereas control animals received a placebo-pellet. Following 10 days to recover from the ovariectomy or implantation of the pellet respectively, mice were anesthetized by intra peritoneal injection of 150 µl 2.5% avertin per 10 g body weight. A skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed by electrical coagulation. The stem of the middle cerebral artery (MCA) was exposed through a burr-hole and was occluded by micro bipolar coagulation (Erbe, Tübingen, Germany). Surgery was performed under a microscope. Mice were kept at a body temperature of 37°C on a heating pad. The body temperature was monitored continuously during the surgery with a rectal thermometer. To determine the infarct volume and to perform immunohistochemistry, mice were sacrificed 48 h after the MCAO. For the isolation of the RNA from the cortex, mice were sacrificed 24 h after the MCAO.

2.12 Isolation of the brains after MCAO

For the analysis of the infarct volume and to perform immunohistochemistry later on, the mice were deeply anesthetized (250 µl 2.5% avertin per 10 g body weight). Median thorakotomie was carried out to expose the heart. The intracardial perfusion was performed with 20 ml Ringer's solution. The perfusion was checked by change in liver colour that turns yellow during perfusion. Head was cut at the atlanto-occipital joint and Brains were removed carefully from the skull and immediately frozen on dry ice.

For the isolation of the RNA from the cortex, the mice were deeply anesthetized (250 µl 2.5% avertin per 10 g body weight).The brain tissue dissection was carried out on normal ice. The brain was cut 3 mm frontal and 5 mm caudal on a brain tissue dissection block to restrict the tissue for later RNA isolation to the penumbra and the ischemic core. The left and right hemispheres were separated with a sharp blade. The remaining cortex was dissected from sub cortical tissue with fine tweezers and was immediately frozen in liquid nitrogen.

2.13 Silverstaining and determination of the infarct volume

Coronal serial sections of the forebrain of mice which underwent a MCAO were prepared like described in 2.7.2. Silver stain technique to determine the infarct volume was performed as described (Neudeck et al., 1997). Following solutions were used:

Silver impregnation solution:

A saturated $LiCO_3$-solution was prepared (ca. 12 mg/ml). The $LiCO_3$-solution was mixed with a 10% $AgNO_3$-solution to form a precipitate. The precipitate was dissolved by drip-wise adding a 25% NH_3-solution. Finally

the solution is diluted 1:6 with H_2O. The silver impregnation solution is sensitive to light.

Developing solution:
6.6 g sodium citrate was solved in 420 ml H_2O. Afterwards 120 ml 37% formaldehyde was added and mixed well. Finally 1.8 g hydroquinone and 90 ml acetone were added and the solution was mixed for 60 min.

2.13.1 Silver staining

Frozen sections were thawed and incubated for 2 min with silver impregnation solution while shaking. Afterwards the slides were washed 6 x 1 min with H_2O. Then, slides were incubated for 3 min with developing solution. Finally the slides were washed 3 x 1 min with H_2O and dried overnight.

2.13.2 Measurement of the infarct volume

Stained sections were scanned at 300 dpi and the infarct area was measured using Scion ImageJ software (Scion, Frederick, MD, USA). The data were exported in Microsoft Excel. The unstained area represents not only the infarct area but also surrounding brain oedema as white area. In order to correct for the oedema portion, the difference of the surface of the left and the right hemisphere was subtracted from the measured silver-negative area (Swanson et al 1990). For the calculation of the whole brain infarct volume, the infarct areas were added and multiplied by the distance between the sections (0.4 mm).

$$Y=U-N+I$$

Y = Corrected infarct area (mm^2)
U = Total area of the contralateral Hemisphere (mm^2)
N = Total area of the ipsilateral Hemisphere (mm^2)
I = Infarct area (mm^2).

2.14 Measurement of the physiological parameters

Arterial blood pressure, pulse and blood gas analysis was carried out before and after ischemia. Mice were kept under avertine anaethesia at a heating pad at 37°C and body temperature was measured by a rectal thermometer. The temperature signal was recorded continuously during the ischemia. For the measurement of blood pressure and pulse in a subgroup of mice, a cannula was inserted into the right femoral artery. The blood samples of 150 µl per mouse were collected in a heparin coated glass capillaries for analysis of arterial blood gas, haemoglobin- and glucose-levels. The catheter was washed with 200µl NaCl solution mixed with 50 IE of heparin before measurement of blood pressure. For laser Doppler measurements, the electrode (P415-205; Perimed, Jarfalla, Sweden) was placed 3 mm lateral and 6 mm posterior to the bregma. Relative perfusion units were determined (Periflux 4001; Perimed, Ja¨rfa¨lla, Sweden).

2.15 RNA isolation and real time PCR analysis

2.15.1 RNA isolation

RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden). All solutions and procedures were done according to the technical bulletin. Cortices for RNA

isolation were prepared like described before (2.12). The frozen cortices were put in RLT-buffer and homogenized using a ultra turrax T8 homogenizer (IKA Werke). While RNA isolation genomic DNA was removed from the lysate using RNase-Free DNase Set (Qiagen, Hilden). The procedure was performed according to the technical bulletin. Isolated RNA was dissolved in 40 µl H_2O and stored at -80°C.

2.15.2 RT-PCR

RT-PCR was performed using SuperScript™ II Reverse Transcriptase kit (Invitrogen) including all buffers and enzymes. RT-PCR mixes were set up as described in the following protocol:

1 µl Oligo$(dT)_{18}$ 500 µg/ml
x µl RNA to achieve a mass of 1 µg
1 µl dNTP mix 10 mM
Add H_2O to a volume of 13 µl

The mixture was heated to 65°C for 5 min and chilled on ice. The following components were added:

4 µl 5 x first strand buffer
2 µl 0.1 M DTT

Following incubation at 42°C for 2 min, 1 µl of SuperScript™ II RT was added and the mixture was incubated at 42°C for 1 h. The reaction was inactivated by incubating the mixture at 70°C for 15 min. The synthesized cDNA was then used for real time PCR analysis.

2.15.3 Real time PCR analysis

All real time PCR primers were obtained from Applied Biosystems (Applera Deutschland GmbH, Darmstadt). Real time PCR primers used for the analysis of expression of the genes of interest:

Gene	Protein	Ordering number
Esr1	ERα	Mm 00433149_m1
Bcl-2	Bcl-2	Mm 00477631_m1
Ptgs2	COX-2	Mm 00478374_m1
Ptger1	EP1	Mm 00443097_m1
Ptger2	EP2	Mm 00436051_m1
Cart	CART	Mm 00489086_m1
Bdnf	BDNF	Mm 00432069_m1
Hprt1	HPRT	Mm 00446968_m1

Real time PCR-mix

2 µl cDNA (equivalent to 1 µg RNA)
1 µl real time PCR primer
10 µl ABgene ABsolute QPCR-mix (AB-1138)
7 µl H_2O

Real time PCR was performed using a Chromo4 real time detector (BioRad).

PCR-programme:

95°C – 15’

For 40 times
95°C – 15’’
60°C – 1’
Reading of the fluorescence signal

Hprt expression was used as a reference to calculate the relative expression of the gene of interest. The following formula was used to calculate the relative expression level of the gene of interest:

2^(PCR cycles of Hprt – PCR cycles of gene of interest) = relative expression of the gene of interest

3. Results

3.1 Analysis of Tie2CreER^{T2}-mediated recombination upon tamoxifen treatment

The cloning of the Tie2CreER^{T2}-transgene and its expression analysis in transgenic mice is described elsewhere (Elzer, J. diploma thesis, University of Heidelberg 2002).

To investigate the Tie2CreER^{T2}-mediated endothelial cell specific recombination upon tamoxifen treatment, Tie2CreER^{T2} mice were mated with transgenic mice containing an eGFP-reportergene (Tie2/RAGE$^{eGFP/+}$). An eGFP-gene without promoter sequences was cloned into the locus of the receptor for advanced glycated end products (RAGE). Exons 2 to 7 of the RAGE gene were flanked by two loxP sites. Upon Cre-mediated recombination the intervening sequences were deleted. The deletion event resulted in the movement of the thymidine kinase (tk) promoter next to

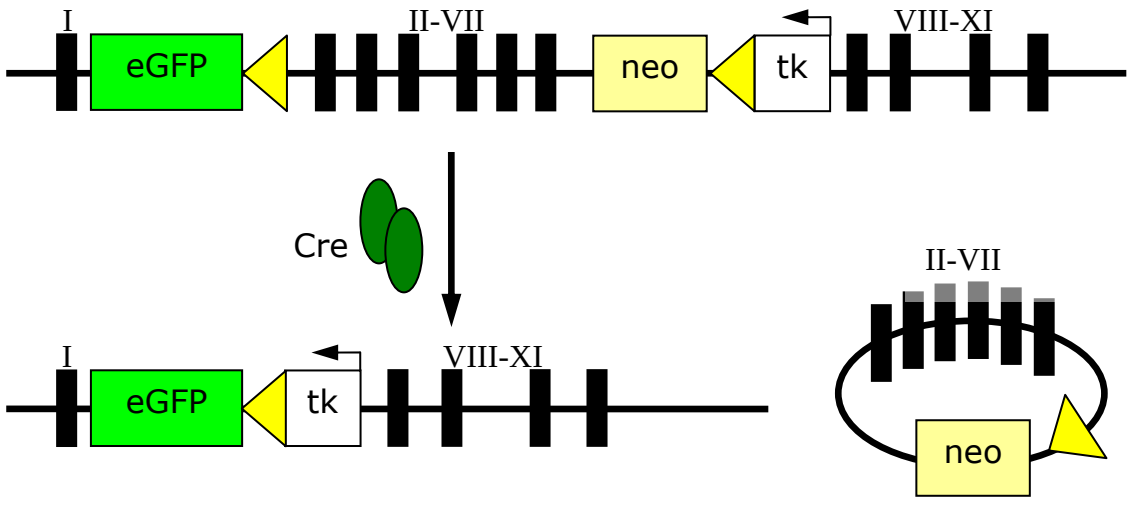

Fig. 3 eGFP expression upon Cre mediated recombination
Cre catalysed recombination leads to deletion of Exon 2 to 7 and the Neo-cassette and to expression of the eGFP-reportergene under control of the tk-promotor.
In cells where recombination has occured the eGFP can be detected by fluorescence microscopy.

the start site of the promotorless eGFP open reading frame (Constien et al., 2001). Consequently, upon Cre recombination, eGFP transcription was activated (Fig. 3).

As illustrated in Fig. 4, eGFP expression was analysed by fluorescence microscopy of 10 µm frozen sections of aorta, liver, brain and kidney of tamoxifen induced and control mice. Frozen sections of the organs of Tie2/RAGE$^{eGFP/+}$-mice without tamoxifen treatment were used as control. In contrast to organs of untreated mice, the endothelial cells of the aorta, the small and bigger veins of the liver, the vessels of the meninges, small vessels of the brain and arteries and peritubular vessels of the kidney of tamoxifen induced Tie2/RAGE$^{eGFP/+}$-mice showed a clear eGFP signal.

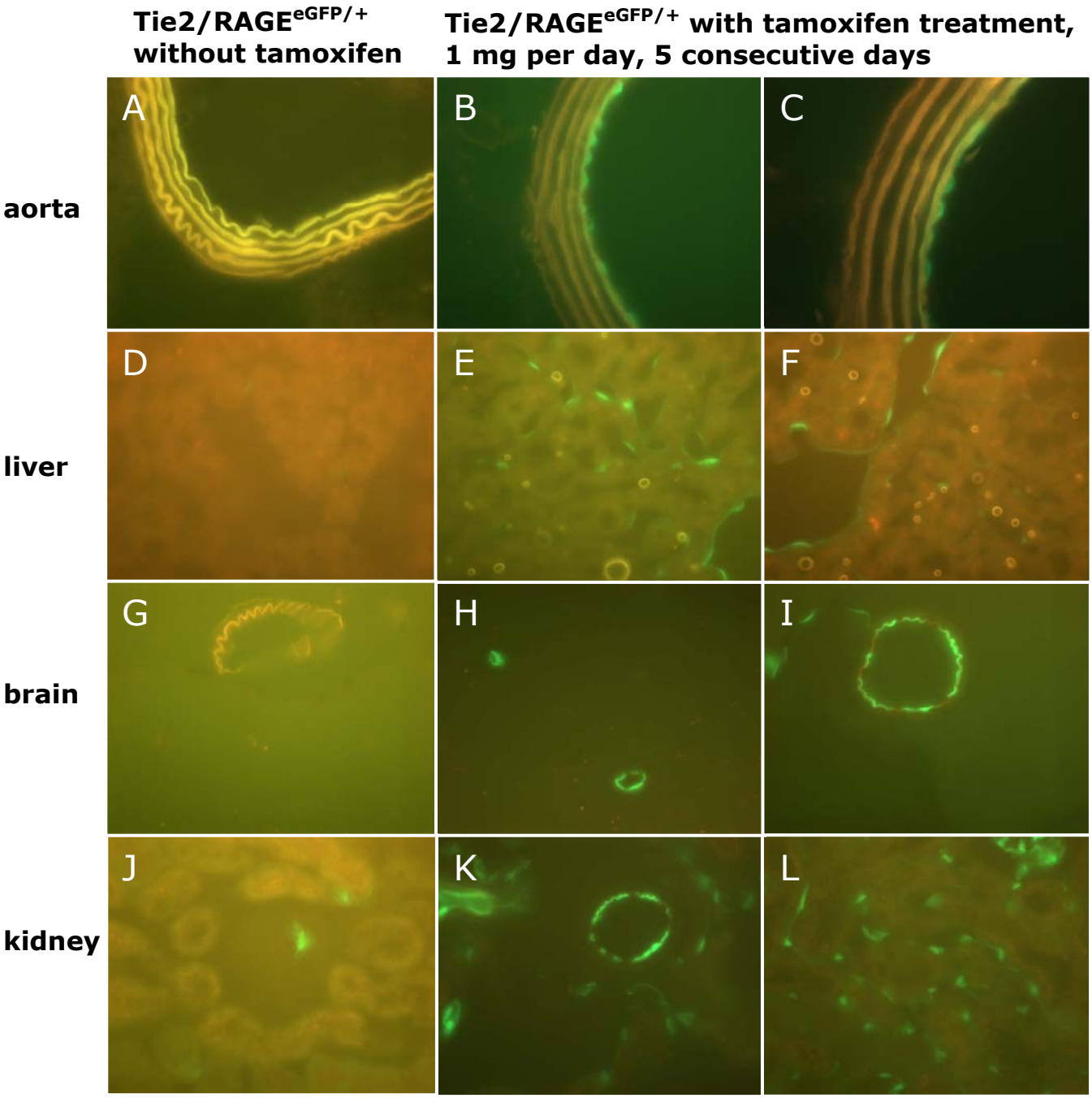

Fig. 4 Tie2CreER^{T2}-mediated recombination upon tamoxifen treatment
Fluorescence microscopy of frozen 10 µm sections of aorta, liver, brain and kidney of Tie2/RAGE$^{eGFP/+}$-mice.
Frozen sections of aorta, liver, brain and kidney of Tie2/RAGE$^{eGFP/+}$-mice without tamoxifen treatment (A, D, G, J) showed no eGFP signal in the endothelial cell layer.
Frozen sections of these organs of Tie2/RAGE$^{eGFP/+}$-mice treated with 1mg tamoxifen per day for 5 consecutive days resulted in Tie2CreER^{T2}-mediated recombination and occurred in eGFP expression in the endothelial cell layer (B, E, H, K, C, F, I, L).

3.2 Immunohistochemical analysis of Tie2CreER^{T2}-mediated deletion of ERα in endothelial cells upon tamoxifen treatment

The analysis of the ERα loss in endothelial cells of Tie2/ER$^{fl/fl}$-mice following tamoxifen treatment, was done by immunohistochemistry on 6 µm paraffin sections of the isolated organs. Paraffin sections of organs from uninduced Tie2/ER$^{fl/fl}$-mice were used as control. Endothelial cells of the aorta, liver and the brain were immunoreactive for ERα in uninduced Tie2/ER$^{fl/fl}$-mice (Fig. 5 A,D,G). The endothelial cells of the aorta of tamoxifen induced Tie2/ER$^{fl/fl}$-mice (Fig. 5 B,C) as well as the endothelial cells of the vessels of the brain (Fig. 5 H,I) showed ERα immunoreactivity. However, the endothelial cells of the small veins showed an ERα loss, whereas endothelial cells of big veins and arteries showed ERα immunoreactivity (Fig. 5 E,F). Since the recombination pattern of ERα in the endothelial cells of tamoxifen induced Tie2/ER$^{fl/fl}$-mice showed a heterogenous pattern these mutants were not used for further investigations about the role of ERα in stroke.

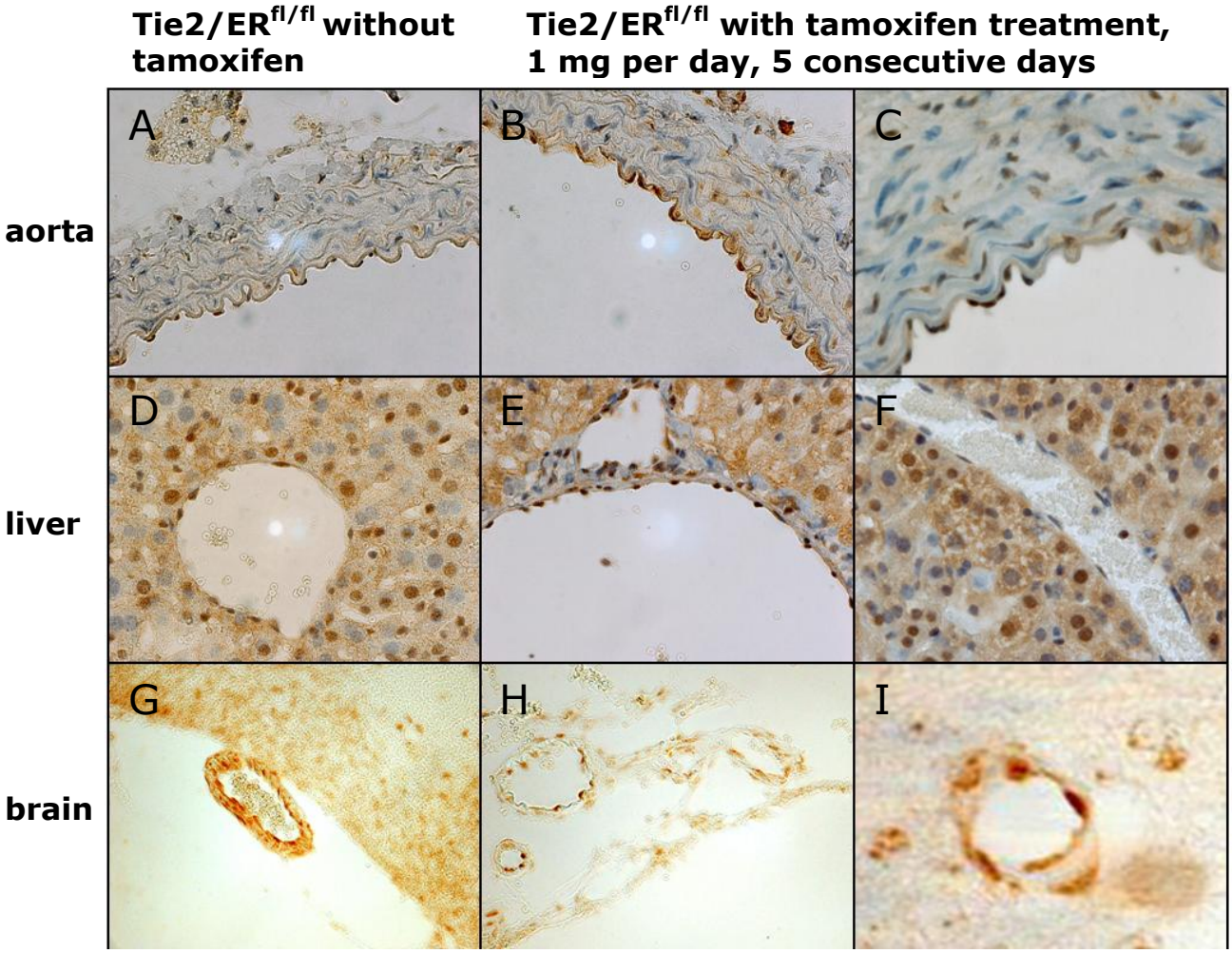

Fig. 5 ERα loss in Tie2/ER$^{fl/fl}$-mice following tamoxifen treatment using immunohistochemistry

ERα positive cells were visualized on 6 µm parafin sections performing DAB staining (brown signals). Additionally, on parafin sections of aorta and liver (Fig. 6 A-F) a hematoxylin counterstain (blue signals) was performed.

Immunohistochemistry of ERα on paraffin sections of uninduced Tie2/ER$^{fl/fl}$-mice (A,D,G). Endothelial cells of the vascular system were all ERα positive (brown signals).

(B,C) Induced Tie2/ER$^{fl/fl}$-mice showed a positive signal for ERα in endothelial cells of the aorta, of the vessels of the meninges (H) and small veins of the brain (I).Additionally endothelial cells of big veins of the liver (E) were ERα positive, too. ERα protein was lost in smaller veins of the liver (F), upon tamoxifen induction.

3.3 Immunohistochemical analysis of ERα deletion in CaMKIICre/ER$^{fl/fl}$-mice in cortical neurons.

ERα deletion in cortical neurons was analysed using immunohistochemistry. Following isolation of brains of CaMKIICre/ER$^{fl/fl}$-mice and ERα$^{fl/fl}$-mice as a control, 20 µm vibratome sections were prepared. Immunohistochemistry for ERα on free floating sections of ERα$^{fl/fl}$-mice revealed an ERα expression in the cortex (Fig. 6 A,C). In contrast no ERα was detectable in the cortex of CaMKIICre/ER$^{fl/fl}$-mice (Fig. 6 B,D). The loss of ERα in the neruons of the cortex is in line with previous findings which showed the loss of ERα in the neurons of the hypothalamus in CaMKIICre/ER$^{fl/fl}$-mice (Wintermantel et al., 2006).

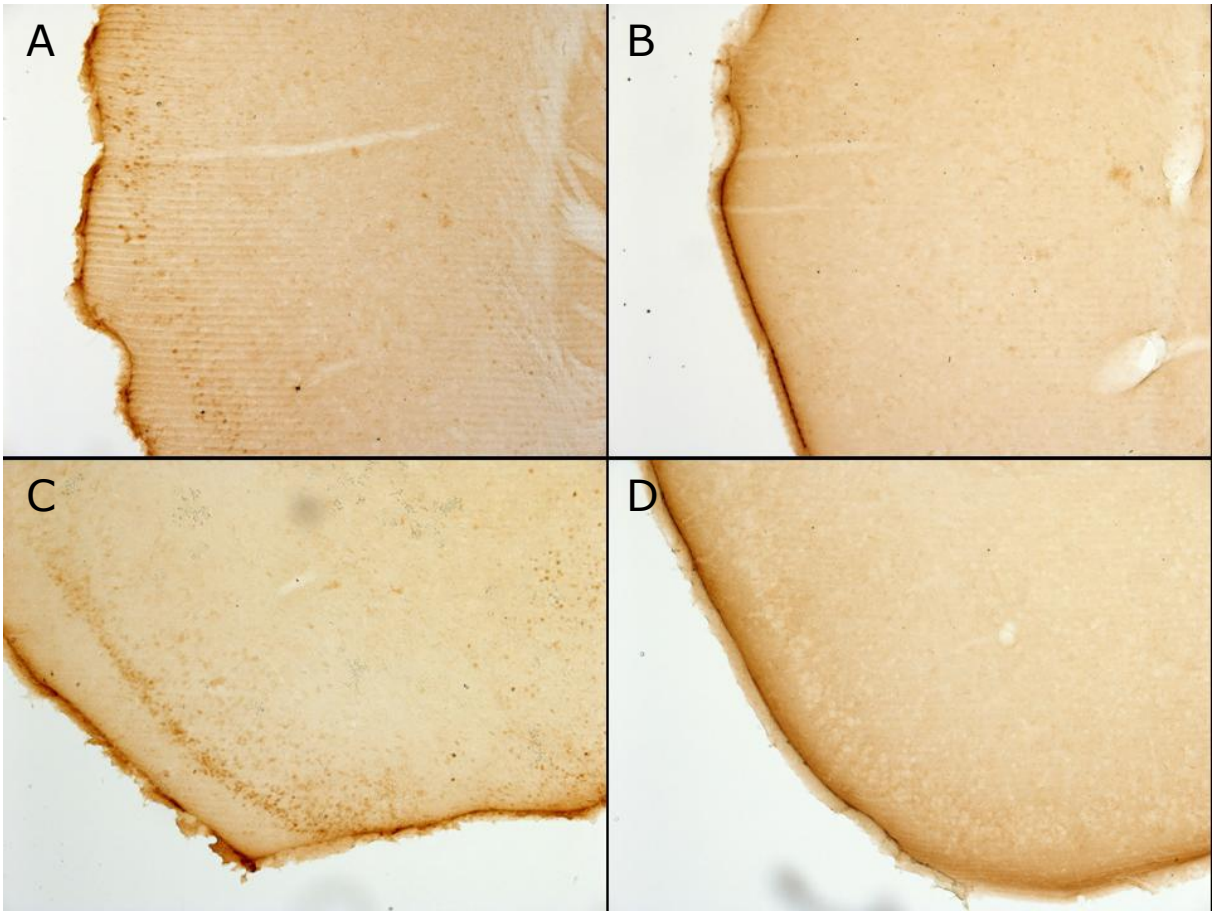

Fig. 6 Analysis of ERα loss in the cortex of CaMKIICre/ER$^{fl/fl}$-mice using immunohistochemistry
On 20 µm freefloating sections of the brain ERα was detected in the ectorhinal cortex (A) and the piriform cortex (C) of ERα$^{fl/fl}$-mice (brown signals). In CaMKIICre/ER$^{fl/fl}$-mice ERα was not detectable in the cortex (B,D).

3.4 Analysis of estradiol effects in stroke

In order to study the role of E_2 in stroke, female wildtype mice underwent a middle cerebral artery occlusion (MCAO). The mice were ovarectomized and received an estradiol pellet (0.025 mg, 21 days release) which results in a constant E_2 level of 35 pg/ml (Horsburgh et al., 2002). Control mice were ovariectomized and received no E_2 pellet. Following 10 days of recovery, the mice underwent a MCAO and were sacrificed after 48 h. The brains were isolated and frozen on dry ice. Serial 20 µm coronal sections every 400 µm of the isolated brains were analysed using silver staining (Fig. 7). Stained sections were scanned and the infarct volume was measured using scion image software (Swanson et al., 1990). As shown in Fig. 8 E_2-treated mice showed a significantly reduced infarct volume of 35% compared to untreated mice (Fig. 8).

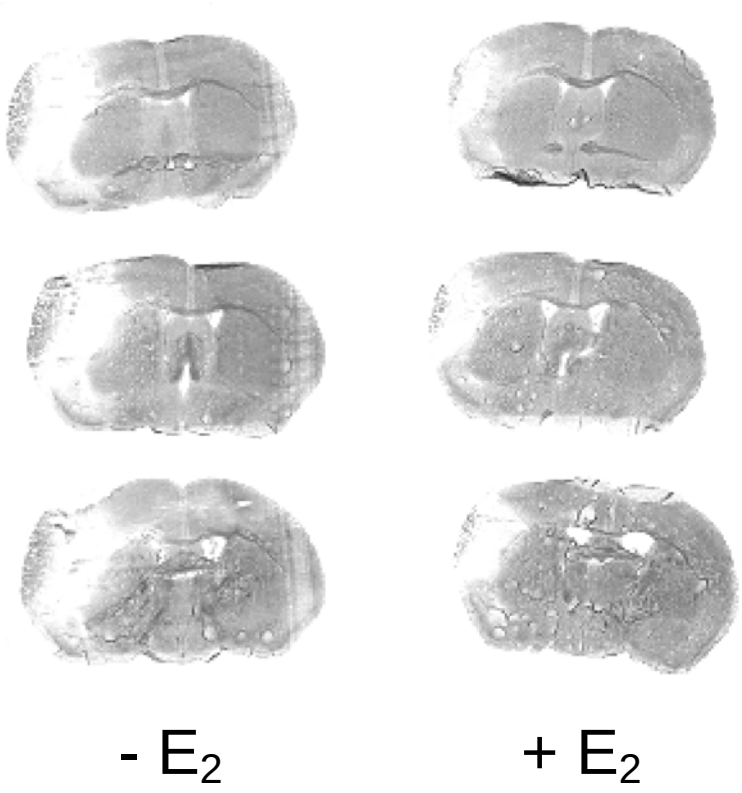

Fig. 7 Silverstaining of coronary brain sections of mice which underwent a MCAO
Typical sections of untreated animals (left side) and E_2 treated animals (right side) are shown. Undamaged and living tissue was silverstained (grey). Apoptotic or necrotic tissue was unstained (white areas).

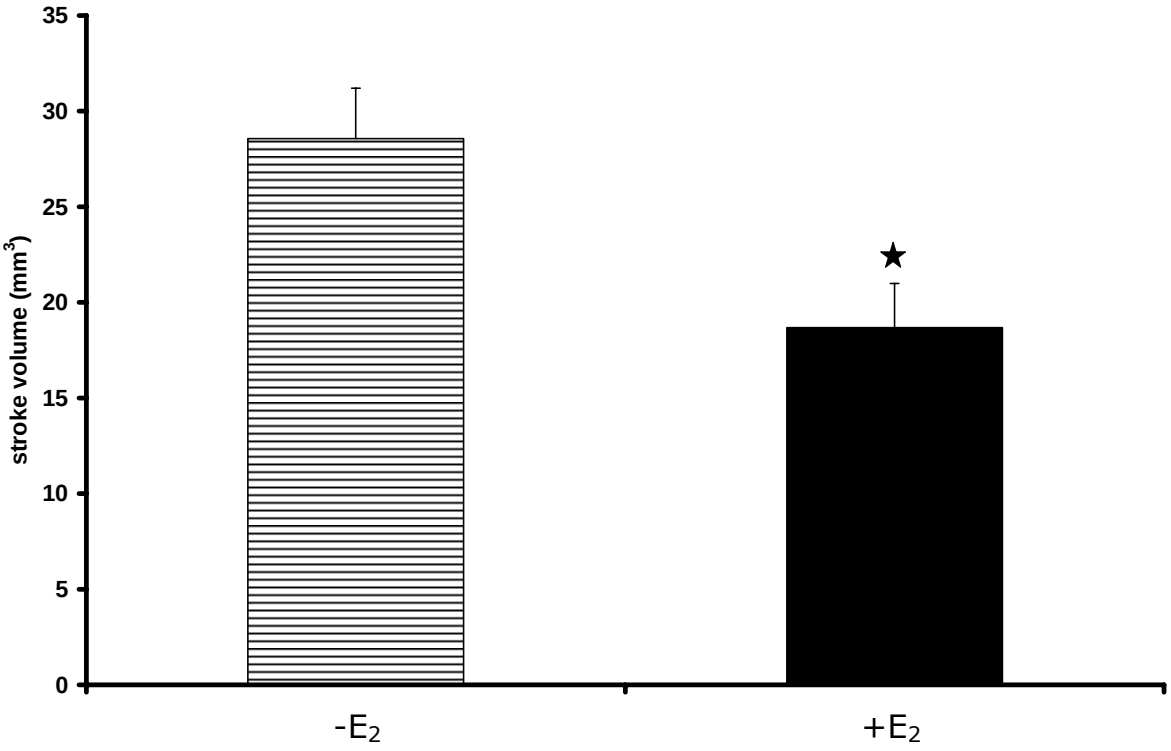

Fig. 8 Quantitative analysis of the stroke volume of untreated and estradiol treated female wildtype mice

Ovariectomized E_2 treated female mice (right bar, n=9) show a clear reduction of the infarct size after 48 h of a MCAO compared to ovariectomized mice without E_2 treatment (left bar, n=6). The reduction of the infarct volume was 35%. ($p<0,03$)

3.5 Analysis of stroke-mediated tissue damage using TUNEL staining on frozen sections from mice which underwent a MCAO

As illustrated in Fig. 7, the area affected by stroke showed massive tissue damage. To examine whether programmed cell death is involved in this tissue damage, a TUNEL staining was performed. Frozen sections from brains of mice, which underwent a MCAO, were prepared and used for a TUNEL staining (Fig. 9) to detect damaged cells. TUNEL positive nuclei were only present in the stroke area (Fig. 9A). To visualize nuclei, a DAPI counterstain was performed (Fig. 9B). Double positive nuclei were represented by light turquoise signals as shown in merged pictures of panels A and B (Fig. 9C).

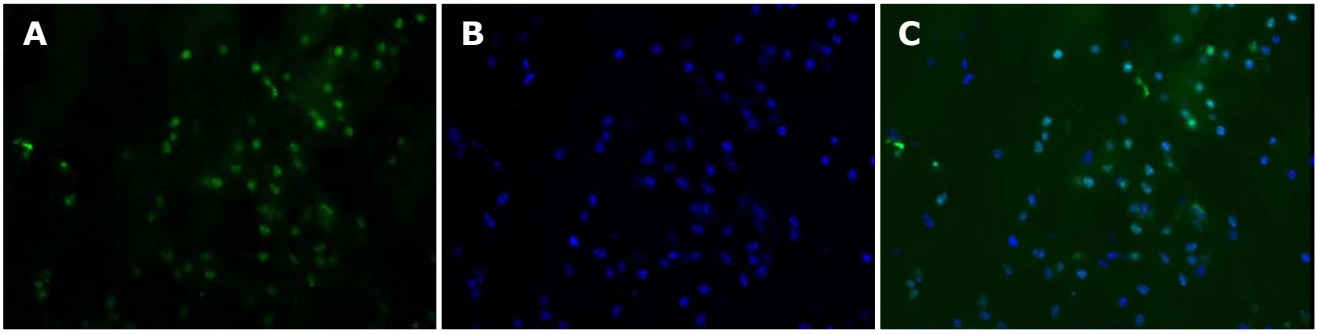

Fig. 9 TUNEL staining on frozen sections from stroke affected brains
Frozen sections from brains of mice which underwent a MCAO, were stained for apoptosis using TUNEL-histochemistry. Sections of E_2 treated mice as well as sections from untreated mice showed TUNEL positive nuclei. Panels A-C show representative pictures of the staining. A) TUNEL positive nuclei (green dots). B) DAPI staining. C) Merged pictures. Double positive nuclei are represented by light turquoise signals.

3.6 Middle cerebral artery occlusion in CaMKIICre/ER$^{fl/fl}$-mice

As shown by Wise and coworkers, E_2 induced neuroprotective effects in a MCAO are mediated through ERα and not ERβ (Dubal et al., 2001). The cell type, however, that receives the estradiol signal and mediates its neuroprotection was not identified. To evaluate the role of neuronal ERα in a MCAO in the presence of E_2, female CaMKIICre/ER$^{fl/fl}$-mice were ovariectomized and received an E_2 pellet. The mice underwent a MCAO. 48 h after the surgery, the brains were isolated and the stroke volume was determined as described above. Ovariectomized ERα$^{fl/fl}$-mice treated with E_2 were used as a control. To estimate the relevance of neuronal ERα in stroke, a second group of untreated ovariectomized ERα$^{fl/fl}$-mice underwent a MCAO.

As it has already been shown in the previous experiment, E_2 reduced the infarct volume in the E_2 treated control group compared to the untreated control group (Fig. 10 left and middle bar). This neuroprotective effect was completely lost in CaMKIICre/ER$^{fl/fl}$-mice despite the fact that these

mice were treated with E_2 (Fig. 10 right bar). The size of the stroke volume was comparable to that of the untreated control group which underwent a MCAO.

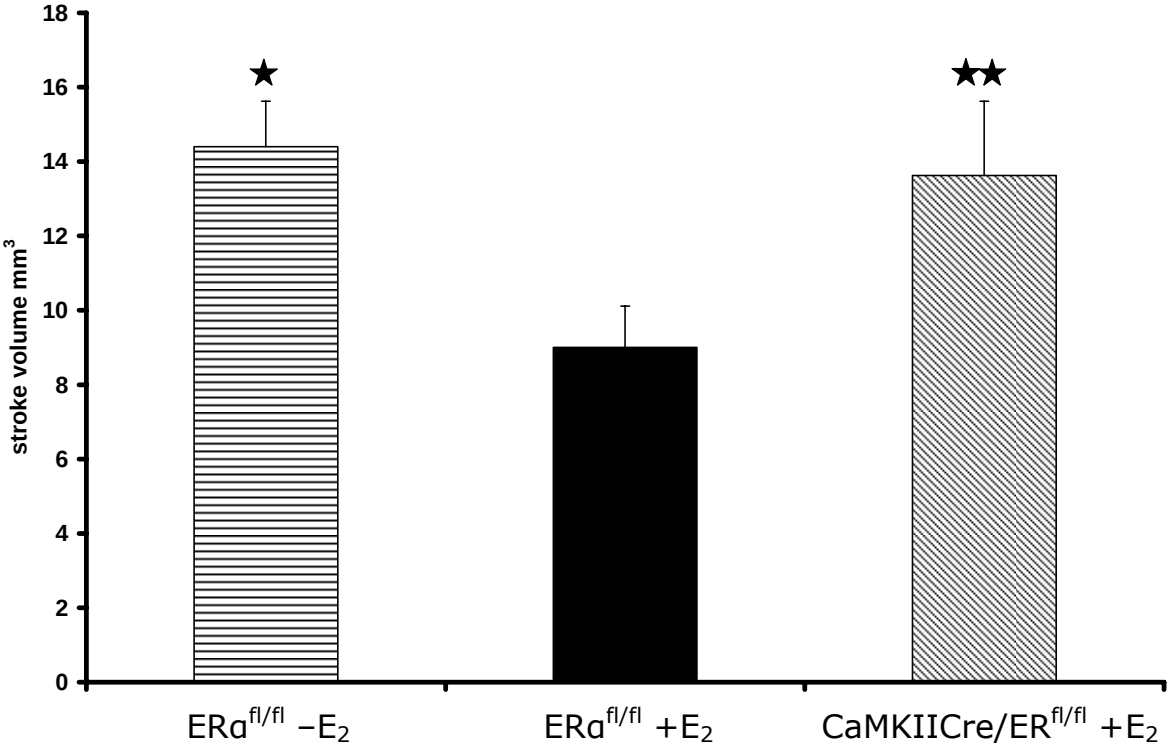

Fig. 10 Quantitative analysis of the stroke volume in female neuronal ERα knock out mice compared to female ERα$^{fl/fl}$-mice untreated and treated with E_2
Quantitative analysis of the stroke volumes of ERα$^{fl/fl}$-mice without E_2-pellet, ERα$^{fl/fl}$-mice with E_2-pellet and CaMKIICre/ER$^{fl/fl}$-mice with E_2-pellet. All mice were ovariectomized. The mice were sacrificed following 48 h of a MCAO and the stroke volume was analysed as described above. The stroke volume of E_2 treated ERα$^{fl/fl}$-mice (middle bar, n=11) was clearly reduced to about 37% compared to untreated ERα$^{fl/fl}$-mice (left bar, n=11, p<0,03) as well as to about 34% compared to the stroke volume of CaMKIICre/ER$^{fl/fl}$-mice (right bar, n=8, p<0,01). The neuroprotective effect of E_2 in stroke was completely lost in the neuronal specific ERα knockout.

Data by Hurn and coworkers suggest that E_2 also has a neuroprotective effect in male rats which underwent a MCAO (Toung et al., 1998). In order to examine this hypothesis in the model used here, and to find out whether E_2 induced neuroprotective effects in a MCAO are also mediated by neuronal ERα in male mice, the previous experiment was repeated as

described above, using E_2 treated male CaMKIICre/ER$^{fl/fl}$-mice and E_2 treated and untreated male ERα$^{fl/fl}$-mice as controls.

As expected, E_2 reduced the stroke volume in male ERα$^{fl/fl}$-mice (n=8) compared to male ERα$^{fl/fl}$-mice without E_2 treatment (n=10, p<0,02) (Fig. 11 left and middle bar). Moreover, consistent with the results in female neuronspecific ERα mutants, the neuroprotective effect of E_2 was lost in male CaMKIICre/ER$^{fl/fl}$-mice (n=11, p<0,01) (Fig. 11 right bar).

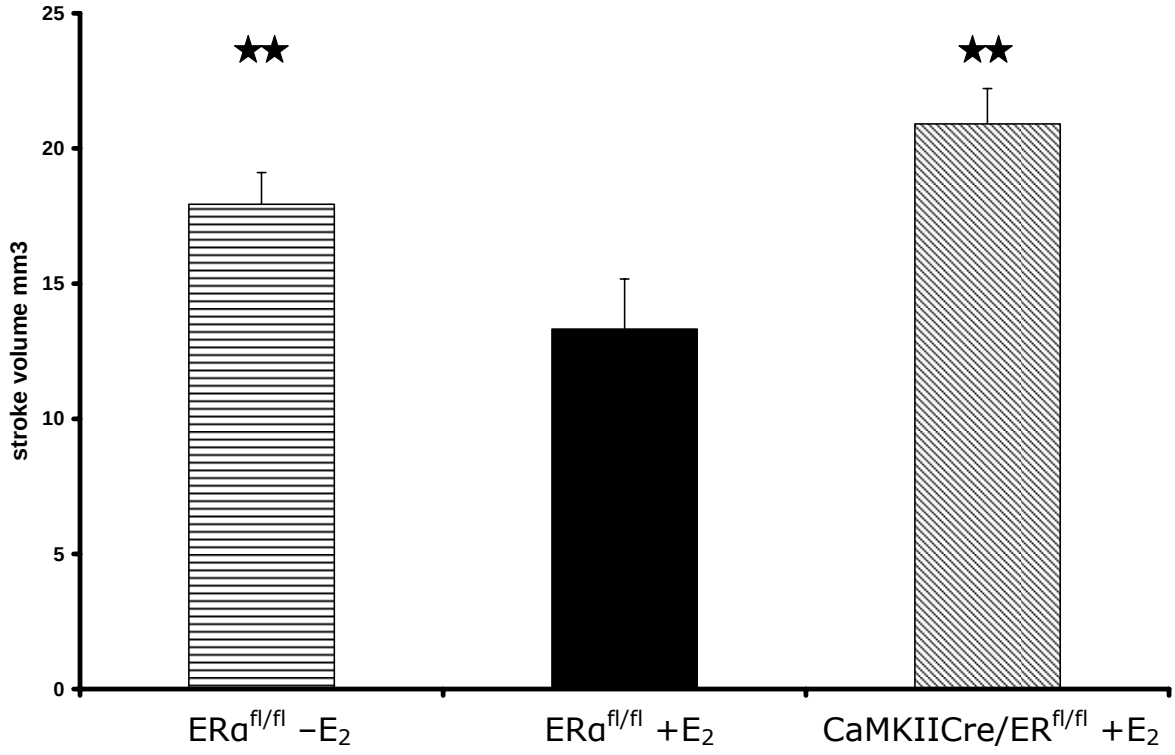

Fig. 11 Quantitative analysis of the stroke volume of male neuronal ERα knock out mice compared to male ERα$^{fl/fl}$-mice untreated and treated with E_2

Quantitative analysis of the stroke volumes of male ERα$^{fl/fl}$-mice without E_2-pellet, male ERα$^{fl/fl}$-mice with E_2-pellet and male CaMKII/ER$^{fl/fl}$-mice with E_2-pellet. The mice underwent a MCAO and were sacrificed following 48 h. The stroke volume of E_2 treated male ERα$^{fl/fl}$-mice (middle bar, n=8) was significantly reduced to about 26% compared to untreated ERα$^{fl/fl}$-mice (left bar, n=10, p<0,02). Consistent with the result of the female neuronal knock out obtained before, the stroke volume of E_2 treated male ERα$^{fl/fl}$-mice is significantly reduced to about 36% compared to E_2 treated CaMKIICre/ER$^{fl/fl}$-mice (right bar, n=11, p<0,01).

3.7 Analysis of the physiological parameters of female CaMKIICre/ER$^{fl/fl}$-mice

The pre-ischemic and post-ischemic physiological parameters of CaMKIICre/ER$^{fl/fl}$-mice (n=4) and ERα$^{fl/fl}$-mice (n=4) both treated with E_2 were analysed. This analysis was done to exclude that the deletion of ERα in the brain leads to secondary effects on cardiovascular physiology that influences stroke. The following physiological parameters were analysed: mean arterial blood pressure, pulse, body temperature, blood flow using Doppler analysis, glucose levels, partial pressure of oxygen, partial pressure of carbon dioxide, ion composition (BE), pH, haemoglobin oxygen saturation, haemoglobin carbon dioxide saturation, share of methaemoglobin and total oxygen concentration (table 1). The physiological analysis showed no significant differences between the pre-ischemic ERα$^{fl/fl}$-mice and the pre-ischemic CaMKIICre/ER$^{fl/fl}$-mice. There were also no significant differences in the physiological parameters of the two groups detectable after performing a MCAO.

Pre-ischemic	**ERα$^{fl/fl}$**	**CaMKIICre/ER$^{fl/fl}$**
Mean arterial pressure (mmHg)	58.5	57.33
Pulse (bpm)	297	296
Body temperature (°C)	37.5	37.23
Blood flow (relative units)	211.5	287.33
Glucose (mg/dl)	262.5	228.67
Partial pressure of O_2 (mmHg)	79.9	82.13
Partial pressure of CO_2 (mmHg)	53.45	49.20
BE (mEq/l)	-6.23	-5.87
pH	7.23	7.25
Haemoglobin O_2 saturation (%)	84.33	82.67
Haemoglobin CO_2 saturation (%)	5.78	5.07
Methaemoglobin concentration (%)	0.93	0.90
Total O_2 concentration (ml/dl)	15	14.47
Post-ischemic		
Mean arterial pressure (mmHg)	54.8	55.67
Pulse (bpm)	306.0	304.00
Body temperature (°C)	37.5	37.43
Blood flow (relative units)	34.8	34.00
Glucose (mg/dl)	279.5	249.00
Partial pressure of O_2 (mmHg)	64.9	76.23
Partial pressure of CO_2 (mmHg)	59.6	53.23
BE (mEq/l)	-9.2	-9.87
pH	7.2	7.10
Haemoglobin O_2 saturation (%)	64.6	76.33
Haemoglobin CO_2 saturation (%)	4.5	4.67
Methaemoglobin concentration (%)	1.1	1.00
Total O_2 concentration (ml/dl)	9.8	12.83

Table 1 Pre- and post-ischemic physiological parameters of ERα$^{fl/fl}$- and CaMKIICre/ERfl/fl-mice, both treated with E_2

The following physiological parameters were analysed: mean arterial blood pressure, pulse, body temperature, blood flow using Doppler analysis, glucose levels, partial pressure of oxygen, partial pressure of carbon dioxide, ion composition (BE), pH, haemoglobin oxygen saturation, haemoglobin carbon dioxide saturation, share of methaemoglobin and total oxygen concentration.

3.8 Analysis of ERα deletion in microglial cells of LysMCre/ER$^{fl/fl}$-mice

Breeding mice expressing the Cre recombinase under control of the lysozyme M promoter to ERα$^{fl/fl}$ mice resulted in a deletion of ERα specific in the monocytic cell lineage including microglial cells. Microglial cells of LysMCre/ER$^{fl/fl}$-mice and ERα$^{fl/fl}$-mice as a control were isolated to determine the recombination efficiency and deletion of ERα in these cells. To show ERα loss in isolated microglial cells, an immunocytochemistry for ERα and isolectin B4 was performed (Fig. 12). ERα appears red in the controls and was mainly restricted to the nucleus of the cells (Fig. 12A), whereas 92% of the microglial cells isolated from LysMCre/ER$^{fl/fl}$-mice showed no ERα signal (Fig. 12B). Cells were counterstained with isolectin B4 appearing green upon fluorescence microscopy, to identify them as microglial cells.

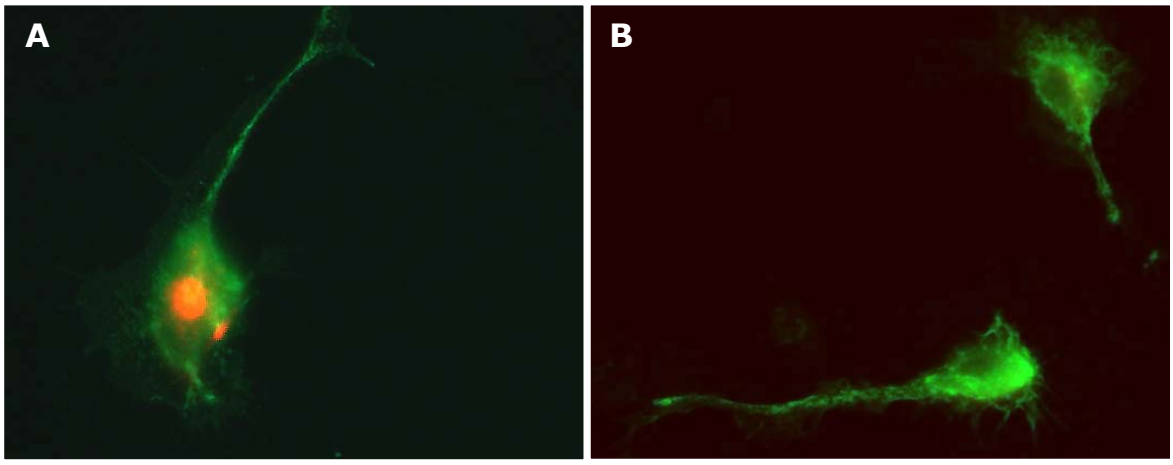

Fig. 12 Quantitative analysis of ERα deletion in microglial cells isolated from LysMCre/ER$^{fl/fl}$-mice
Microglial cells from ERα$^{fl/fl}$-mice as a control (Fig. A) and from LysMCre/ER$^{fl/fl}$-mice (Fig. B) were isolated and a immunocytochemistry for ERα and isolectin B4 were performed. ERα positive cells show a red signal upon fluorescence microscopy, whereas isolectin B4 positive cells appear green.

3.9 Middle cerebral artery occlussion in LysMCre/ER$^{fl/fl}$-mice

Since it has been proven that ERα in neurons plays a critical role in mediating neuroprotective effects of E_2, the role of ERα in microglial cells in stroke was investigated using LysMCre/ER$^{fl/fl}$-mice. The experiment was performed as described above. Three groups of female mice were ovariectomized and underwent a MCAO. ERα$^{fl/fl}$-mice received no estradiol pellets, whereas a second ERα$^{fl/fl}$-group received an E_2-pellet. Additionally LysMCre/ER$^{fl/fl}$-mice received an E_2-pellet. The MCAO experiment was performed as described above.

In agreement with previous MCAO experiments the neuroprotective effect of E_2 was unequivocally displayed between E_2 treated and control ERα$^{fl/fl}$-mice (Fig.13 left and middle bar, n=11, p<0,01). There was also a clear visible neuroprotective effect of E_2 in female LysMCre/ER$^{fl/fl}$-mice compared to ERα$^{fl/fl}$ control mice (Fig. 13 right bar, n=8, p<0,01). This was in contrast to the previous MCAO experiments where the neuroprotective effect of E_2 was lost in the neuronal specific ERα knock out.

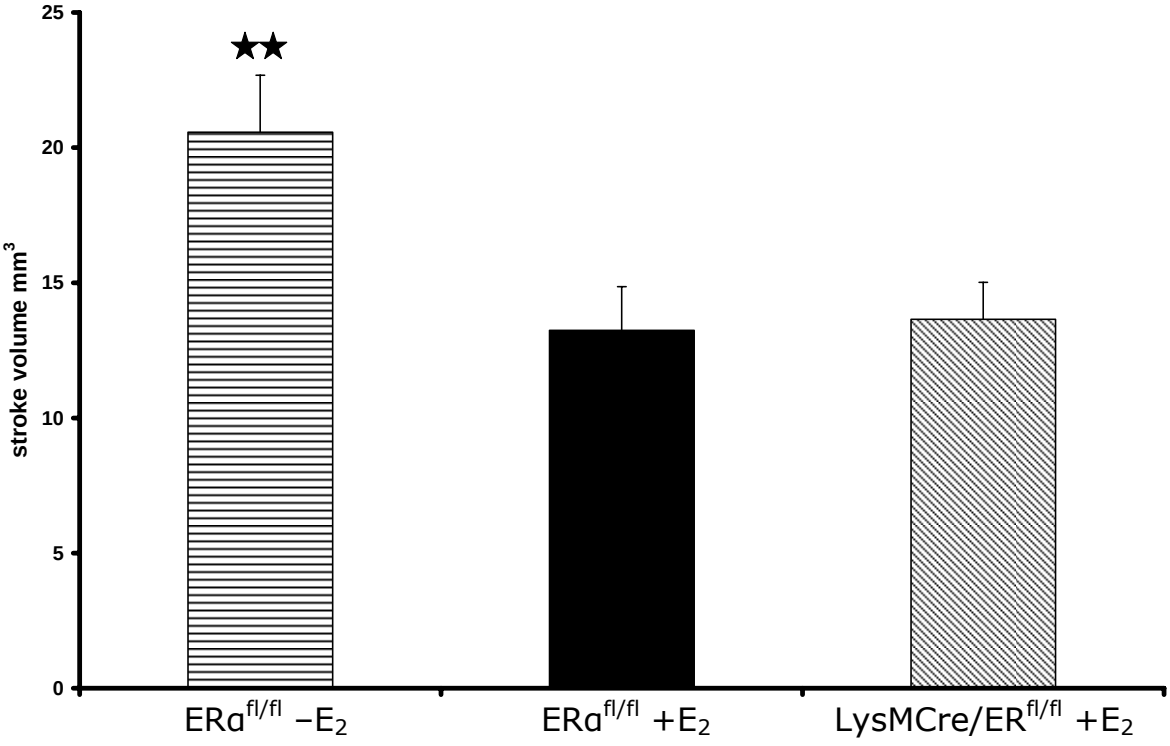

Fig. 13 Quantitative analysis of the stroke volume of female microglial ERα knock out mice compared to female ERα$^{fl/fl}$-mice untreated and treated with E_2
Quantitative analysis of the stroke volume of female ERα$^{fl/fl}$-mice without E_2-pellet, female ERα$^{fl/fl}$-mice with E_2-pellet and female LysMCre/ER$^{fl/fl}$-mice with E_2-pellet. The mice underwent a MCAO and were sacrificed following 48 h. The stroke volume of E_2 treated ERα$^{fl/fl}$-mice (middle bar, n=11) was significantly reduced to about 36% compared to untreated ERα$^{fl/fl}$-mice (left bar, n=11, p<0,01). In contrast to the previous CaMKIICre/ER$^{fl/fl}$-experiments, the stroke volume of E_2 treated female LysMCre/ER$^{fl/fl}$-mice was reduced (n=8, p<0,01).

3.10 Real time PCR expression analysis of RNA isolated from cortices of female CaMKIICre/ER$^{fl/fl}$-mice which underwent a MCAO

For the analysis of ERα dependent transcriptional regulation in a MCAO, female CaMKIICre/ER$^{fl/fl}$-mice and ERα$^{fl/fl}$-mice as controls underwent a MCAO. As described in the previous sections, the mice were ovarectomized and received an E_2-pellet. As a control, one group of ERα$^{fl/fl}$-mice were left untreated.

The stroke volume reaches its maximum size after 48 h. Upon this time point, no more expansion of the stroke volume could be detected. To monitor the neuroprotective events on a transcriptional level, the mice were sacrificed 24 h following a MCAO. At that time, expression of genes participating in apoptotic or antiapoptotic events, is strongly changed (Alkayed et al., 2001; Dubal et al., 2006). Therefore cortices of the mice were prepared at 24 h of MCAO and immediately frozen in liquid nitrogen. The RNA of the tissues was isolated and a RT-PCR was performed. The resulting cDNA was then used for further expression studies by real time PCR as displayed in the following sections. Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) was used as a reference gene for all real time PCR experiments in this study to quantify the changes in the RNA-expression levels of the investigated genes (Meldgaard et al., 2006).

3.10.1 Expression levels of ERα in stroke

As demonstrated before, the neuroprotective effects of E_2 in neurons are mediated via ERα. Since it has been shown, that a MCAO can induce the upregulation of ERα expression in the brain (Dubal et al., 2006), $ERα^{fl/fl}$-mice were analysed for MCAO dependent transcriptional regulation of ERα. ERα expression levels in cortices affected by a MCAO (ipsilateral) were compared to the expression levels of ERα in unaffected cortices (contralateral)(Fig. 14).

In absence of E_2 transcription of ERα was sifnificantly higher in ipsilateral cortices than in the contralateral cortices (Fig.14 two left bars, n=9, p<=0,02). These findings were consistent with the result obtained on the cortices of E_2 treated mice (Fig.14 two right bars, n=10, p<0,03). ERα transcription was significantly elevated in the ipsilateral cortices compared to contralateral cortices of E_2 treated mice.

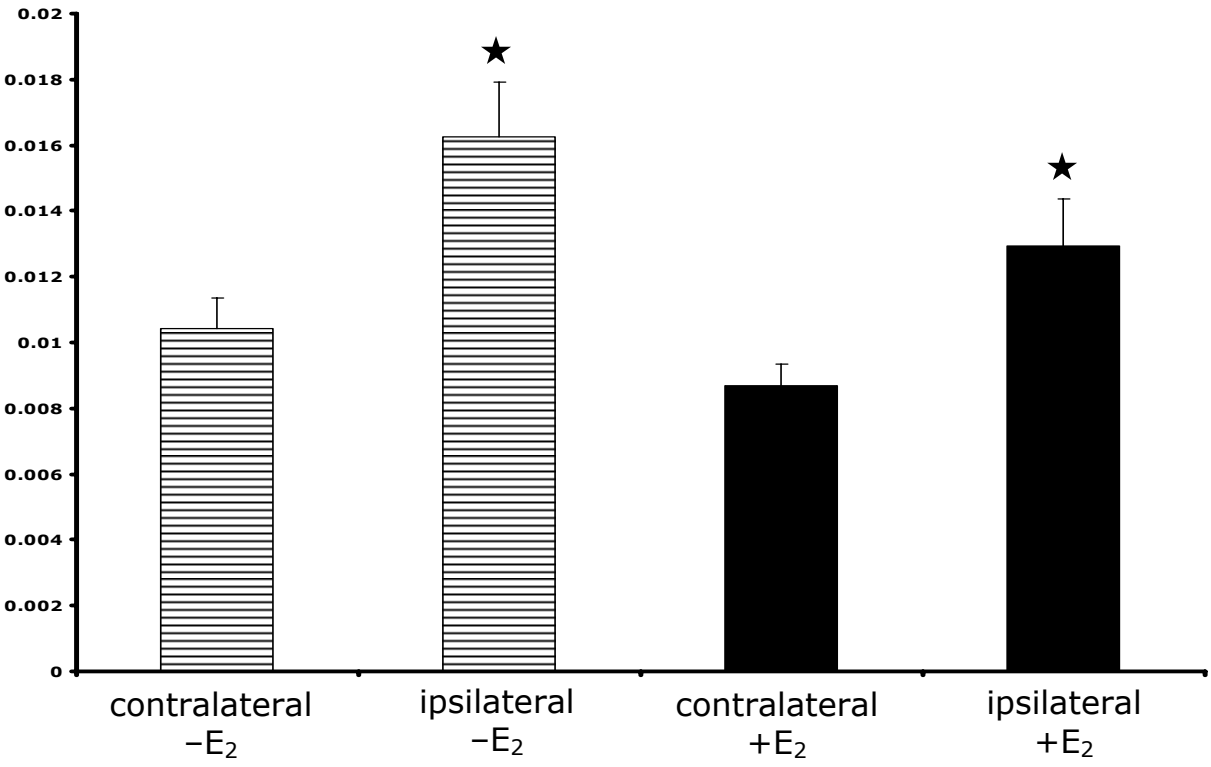

Fig.14 Analysis of ERα expression levels in ipsilateral and contralateral cortices in MCAO

Real time PCR analysis of ERα expression in ipsilateral cortices compared to contralateral cortices of E_2 treated and untreated ERα$^{fl/fl}$-mice after 24 h of MCAO. ERα expression was measured by taqman analysis. In ispilateral cortices of mice in absence of E_2 (right striped bar, n=9, p<0,02), as well as in the presence of E_2 (right filled bar, n=10, p<0,03), ERα expression was significantly elevated as compared to cortices of the contralateral side.

3.10.2 Expressionanalysis of Bcl-2 in CaMKIICre/ER$^{fl/fl}$-mice following 24 h of a MCAO

It has been shown, that the neuroprotective properties of Bcl-2 in *in vitro* as well as in *in vivo* models of stroke are influenced by E_2 (Choi et al., 2004; Nilsen and Diaz Brinton, 2003; Zhao et al., 2004).

To analyse the Bcl-2 expression level in stroke with respect to E_2 and ERα function in a stroke-model a real time PCR was performed. Two groups of female ERα$^{fl/fl}$-mice, one with E_2-pellets (n=10) and one without (n=9), and an E_2 treated group of female CaMKIICre/ER$^{fl/fl}$-mice (n=9) were

analysed by performing a real time PCR. The RNA was isolated from the ipsilateral cortices of these mice following 24 h of a MCAO. After performing a reverse transcription, the cDNA was used for real time PCR analysis.

In contrast to previous findings (Alkayed et al., 2001; Nilsen and Diaz Brinton, 2003), there were no significant differences in the expression levels of Bcl-2 in the three experimental groups detectable (Fig. 15). Neither an E_2 mediated Bcl-2 regulation (Fig. 15 left and middle bar) nor an ERα dependent Bcl-2 expression could be observed (Fig. 15 middle and right bar), suggesting that Bcl-2 expression is not affected by E_2 or its receptor ERα in this model of stroke.

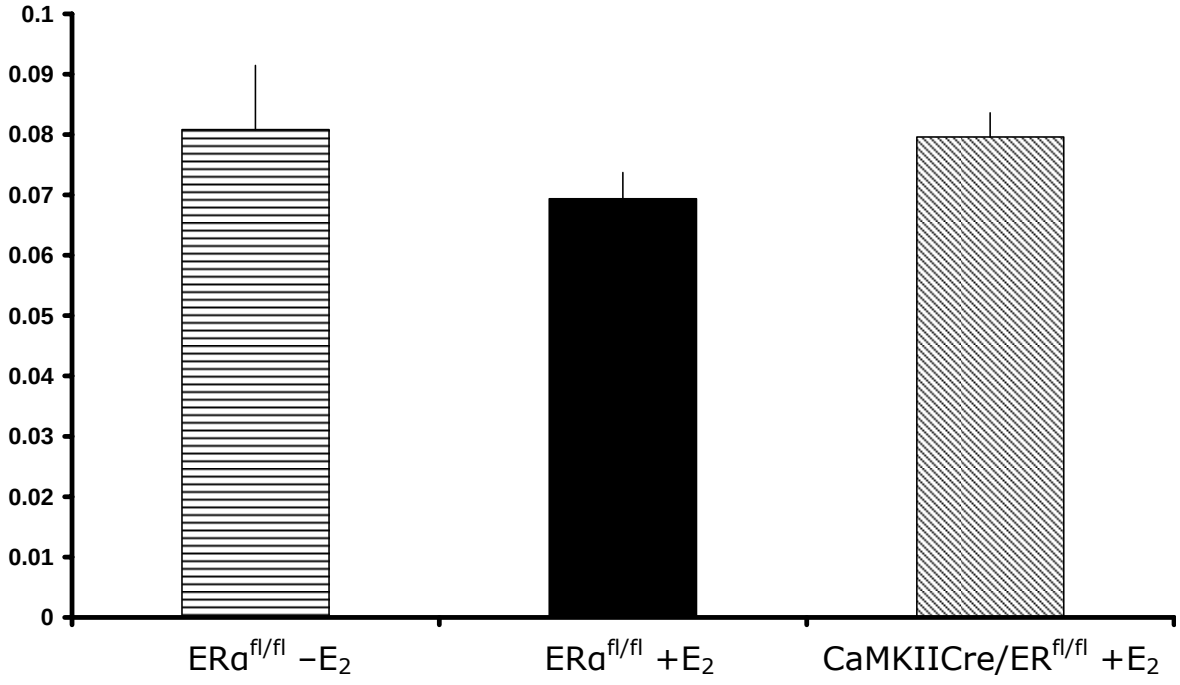

Fig. 15 Bcl-2 expression levels in female CaMKIICre/ER$^{fl/fl}$-mice performing real time PCR analysis

The Bcl-2 expression level was analyzed using a real time PCR technique. RNA from the ipsilateral cortices of ERα$^{fl/fl}$-mice in absence (left bar) and presence (middle bar) of E_2 and CaMKIICre/ER$^{fl/fl}$-mice treated with E_2 (right bar) was isolated following 24 h of a MCAO.

3.10.3 Expressionanalysis of cyclooxygenase-2 in CaMKIICre/ER$^{fl/fl}$-mice after 24 h of a MCAO

Cyclooxygenase-2 (COX-2) catalyses the first step in the synthesis of prostanoids, a large family of arachidonic acid metabolites comprising prostaglandins, prostacyclin, and thromboxanes. COX-2 activity is described to exacerbate neuronal death in ischemia (Wu Chen et al., 2004). In contrast to these observations, COX-2 is required for the development of sexual behaviour in newborn male mice and is upregulated upon estradiol treatment (Amateau and McCarthy, 2004).
Therefore the expression of COX-2 was analysed by real time PCR. The settings and realization of the experiment were conducted as described in section 3.10.2.
The COX-2 expression level in ERα$^{fl/fl}$-mice (n=10) with E_2-pellet was not significantly altered compared to ERα$^{fl/fl}$-mice (n=9) without E_2-pellet (Fig. 16 left and middle bar). However, COX-2 expression was significantly increased in CaMKIICre/ER$^{fl/fl}$-mice (n=9) compared to E_2 treated ERα$^{fl/fl}$-mice (Fig. 16 middle and right bar) suggesting, that ERα is able to suppress the expression of COX-2.

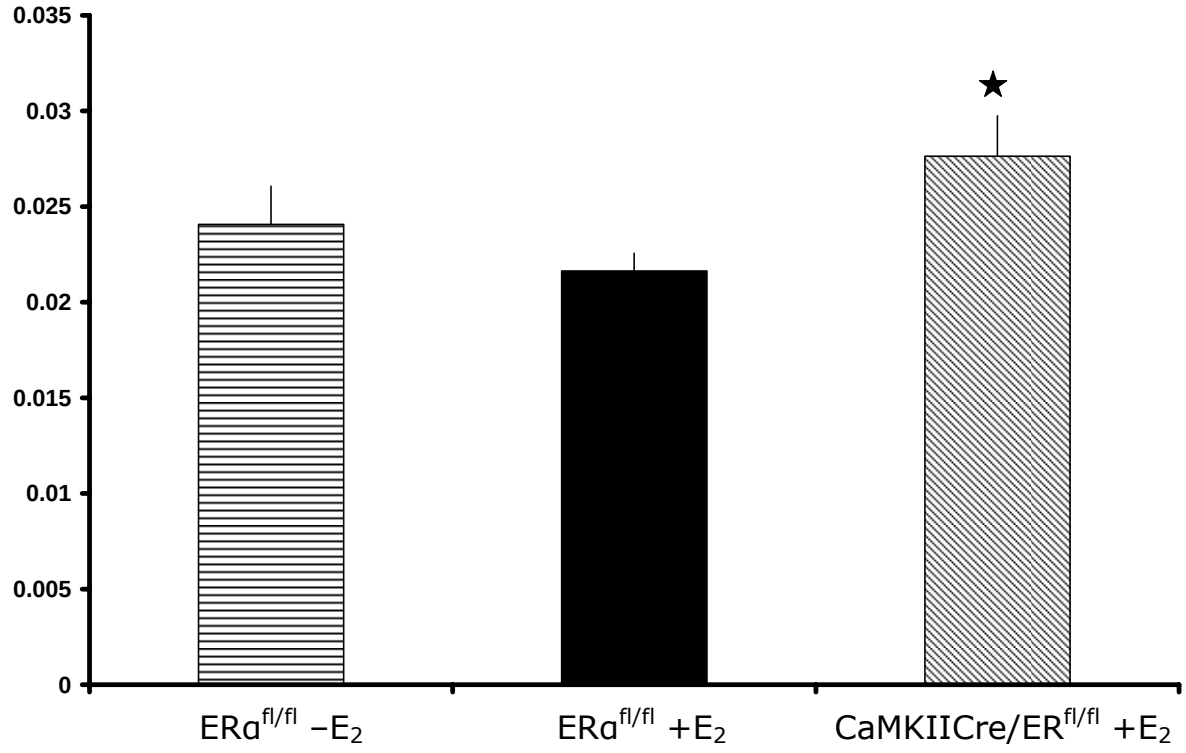

Fig. 16 COX-2 expression levels in female CaMKIICre/ER$^{fl/fl}$-mice using real time PCR analysis
COX-2 mRNA expression was analysed performing a real time PCR. The RNA was isolated from ipsilateral cortices of ERα$^{fl/fl}$-mice lacking E_2 treatment (left bar, n=9) and ERα$^{fl/fl}$-mice (middle bar, n=10) and CaMKIICre/ER$^{fl/fl}$-mice (right bar, n=9) both treated with E_2, after 24 h of a MCAO.

3.10.4 Expressionanalysis of prostaglandin E_2 EP1 receptor (EP1) and prostaglandin E_2 EP2 receptor (EP2) in CaMKIICre/ER$^{fl/fl}$-mice after 24 h of a middle cerebral artery occlusion

The neurotoxic effect of COX-2 is mediated via one of its products prostaglandin E_2 (PGE_2). PGE_2 binds and activates EP1, resulting in the disruption of the Ca^{2+} homeostasis in neurons by disrupting Na^+-Ca^{2+} exchange. In case of an ischemic insult, elevated Ca^{2+} accumulation can lead to increased neuronal damage (Kawano et al., 2006).

In contrast to these findings PGE_2 also binds to EP2. This receptor was postulated to have neuroprotective effects in an ischemic insult (McCullough et al., 2004).

Since COX-2 expression was slightly increased in the CaMKIICre/$ER^{fl/fl}$-mice, reflecting the increased tissue damage in these mice, it was most intriguingly to analyze changes in transcription levels of EP1 and EP2. Isolated RNA of $ER\alpha^{fl/fl}$-mice in presence and absence of E_2 and RNA of CaMKIICre/$ER^{fl/fl}$-mice were used to perform a real time PCR to monitor the levels of EP1 and EP2 transcription. The settings and realization of the experiment were conducted as described in section 3.10.2.

Transcription of EP1 was slightly but not significantly decreased in $ER\alpha^{fl/fl}$-mice treated with E_2 compared to $ER\alpha^{fl/fl}$-mice without E_2 treatment as well as in CaMKIICre/$ER^{fl/fl}$-mice (Fig. 17). Moreover, the transcription levels of EP2 were completely unaffected by E_2 treatment or the loss of neuronal ERα (Fig. 18) when comparing the different experimental groups with each other.

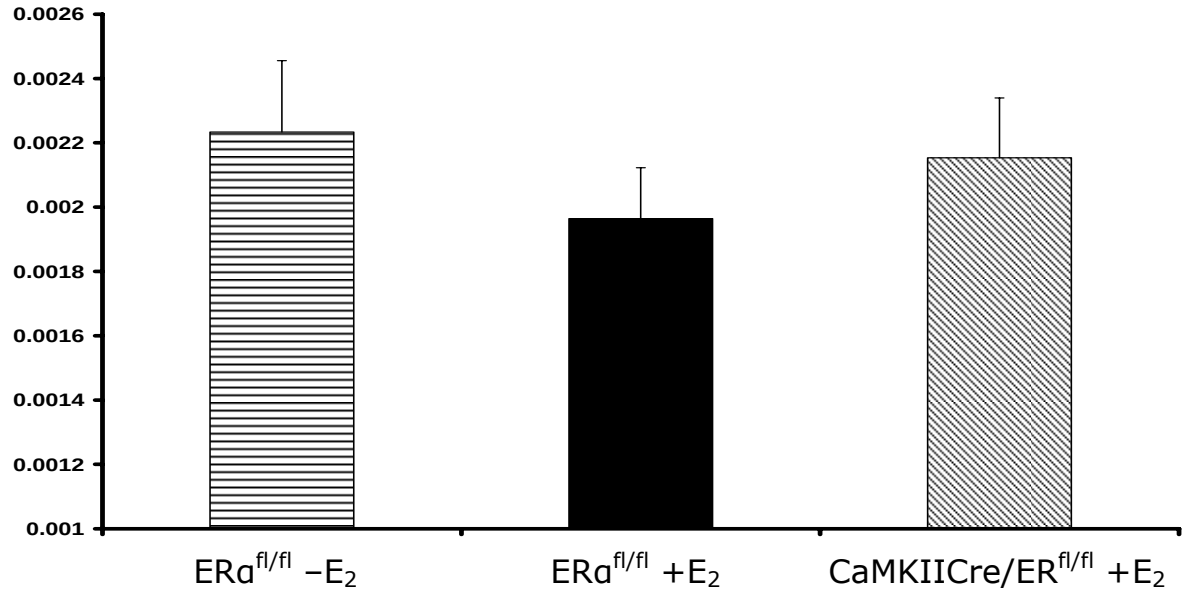

Fig. 17 EP1 expression in female CaMKIICre/$ER^{fl/fl}$-mice using real time PCR analysis

The EP1 expression level was analyzed using a real time PCR technique. RNA from the ipsilateral cortices of $ER\alpha^{fl/fl}$-mice in absence (left bar) and presence (middle bar) of E_2 and CaMKIICre/$ER^{fl/fl}$-mice treated with E_2 (right bar) was isolated following 24 h of a MCAO.

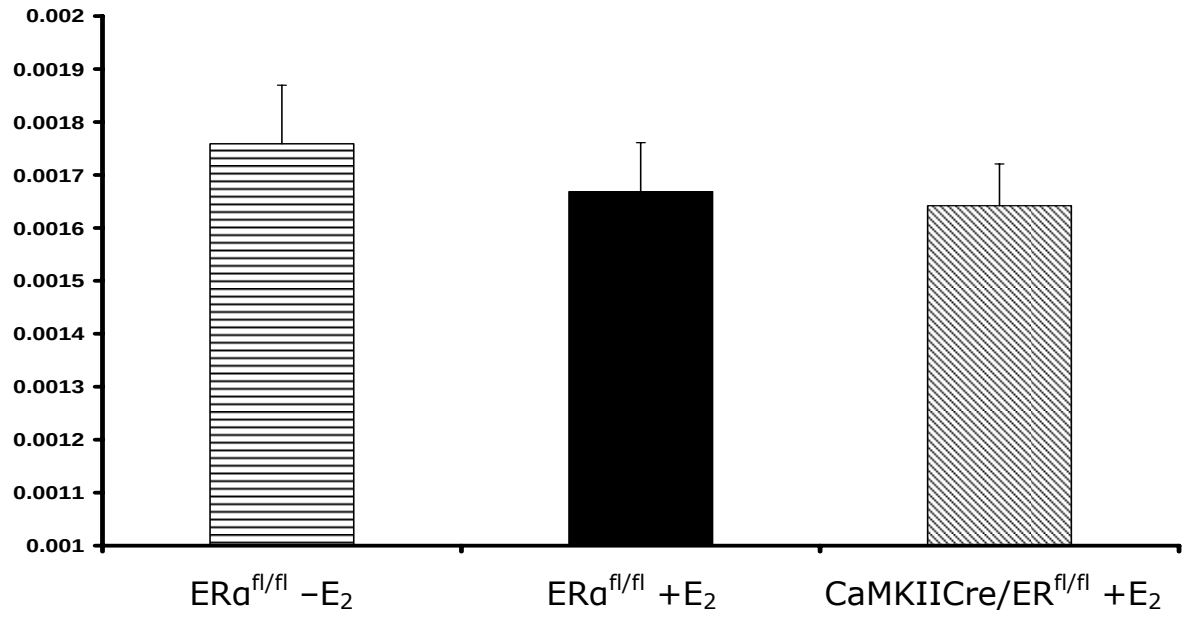

Fig. 18 EP2 expression levels in female CaMKIICre/ER$^{fl/fl}$-mice using real time PCR analysis
Real time PCR analysis of EP2 expression of RNA isolated from the ipsilateral cortices of ERα$^{fl/fl}$-mice without E_2 treatment (left bar, n=9), ERα$^{fl/fl}$-mice (middle bar, n=10) and CaMKIICre/ER$^{fl/fl}$-mice (right bar, n=9) with E_2 treatment. The RNA of the ipsilateral cortices was isolated after the mice underwent a MCAO of 24 h.

3.10.5 Expressionanalysis of cocaine- and amphetamine-regulated transcript (CART) in CaMKIICre/ER$^{fl/fl}$-mice after 24 h of a middle cerebral artery occlusion

Cocaine- and amphetamine-regulated transcript (CART) peptides are neurotransmitters with important roles in a number of physiologic processes. As a modulator of the mesolimbic system, CART is well known to play a role in drug abuse. Additionally, as recently reported, CART has neuroprotective effects in stroke and its expression is inducible by E_2 (Kuhar et al., 2005). To study the expression of CART in stroke and its regulation by E_2, a real time PCR for CART transcription was performed. The experimental settings and procedures were conducted as described in section 3.10.2.

There were no significant differences detectable comparing real time PCR performed on RNA from ERα$^{fl/fl}$-mice with E_2 treatment to ERα$^{fl/fl}$-mice without E_2 treatment (Fig. 19 left and middle bar). There were also no

changes in the transcription level of CART in E_2 treated $ER\alpha^{fl/fl}$-mice compared to E_2 treated CaMKIICre/$ER^{fl/fl}$-mice (Fig. 19 middle and right bar).

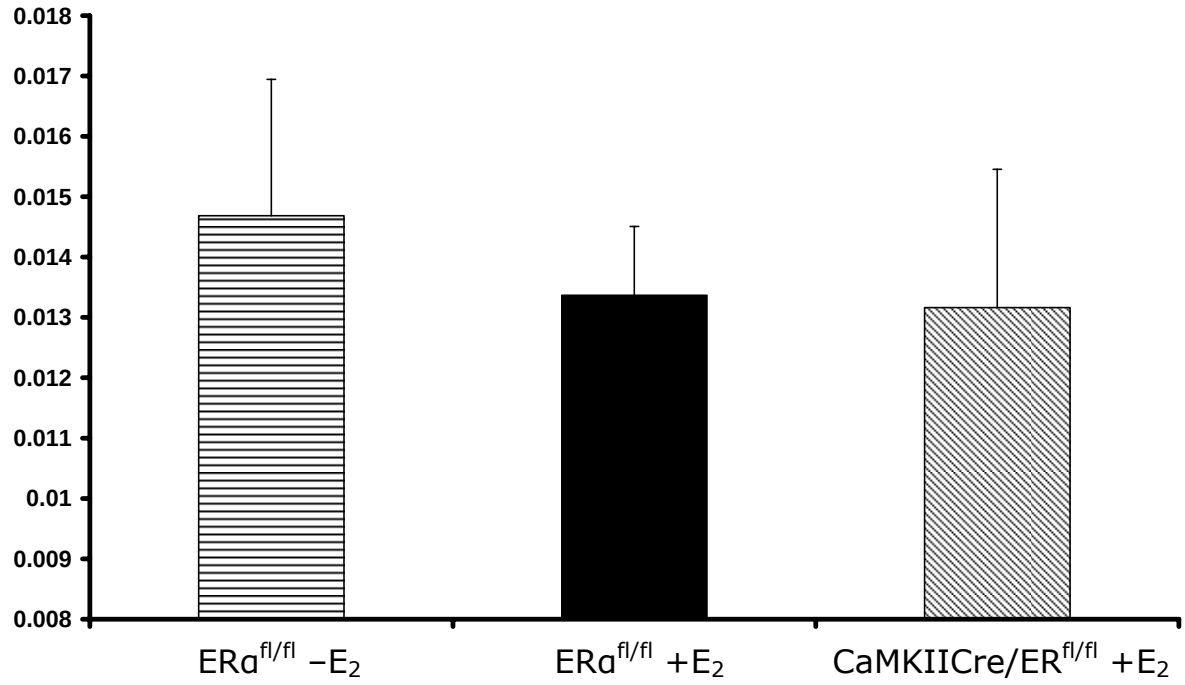

Fig. 19 Analysis of CART expression in female CaMKIICre/$ER^{fl/fl}$-mice performing real time PCR
Real time PCR analysis of CART expression of RNA isolated from the ipsilateral cortices of $ER\alpha^{fl/fl}$-mice without E_2 treatment (left bar, n=9), $ER\alpha^{fl/fl}$-mice (n=10) and CaMKIICre/$ER^{fl/fl}$-mice (n=9) with E_2 treatment (middle and right bar). The RNA of the ipsilateral cortices was isolated after the mice underwent a MCAO for 24 h. The transcription level of CART was not altered in $ER\alpha^{fl/fl}$-mice with E_2-pellet compared to $ER\alpha^{fl/fl}$-mice without E_2-pellet, nor was it changed compared to CaMKIICre/$ER^{fl/fl}$-mice with E_2-pellet.

3.10.6 Analysis of the expression level of brain derived neurotrophic factor (BDNF) in CaMKIICre/$ER^{fl/fl}$-mice after 24 h of a MCAO

It is well known, that E_2 can induce trophic factors in the brain, which promote cell survival in stroke (Wise et al., 2001). Therefore, BDNF transcription was analysed in ovariectomized $ER\alpha^{fl/fl}$-mice in absence and

presence of E_2, as well as in E_2 treated CaMKIICre/ER$^{fl/fl}$-mice, using real time PCR technique. The experimental settings and procedures were conducted exactly as in section 3.10.2.

As illustrated in Fig. 20, BDNF transcription was twofold increased in ERα$^{fl/fl}$-mice treated with E_2 compared to ERα$^{fl/fl}$-mice in absence of E_2 (left and middle bar). Surprisingly, BDNF transcription was more than twofold increased in CaMKIICre/ER$^{fl/fl}$-mice treated with E_2 compared to ERα$^{fl/fl}$-mice in absence of E_2 (Fig 20 middle and right bar). These results suggest that BDNF is upregulated by E_2 independent of neuronal ERα.

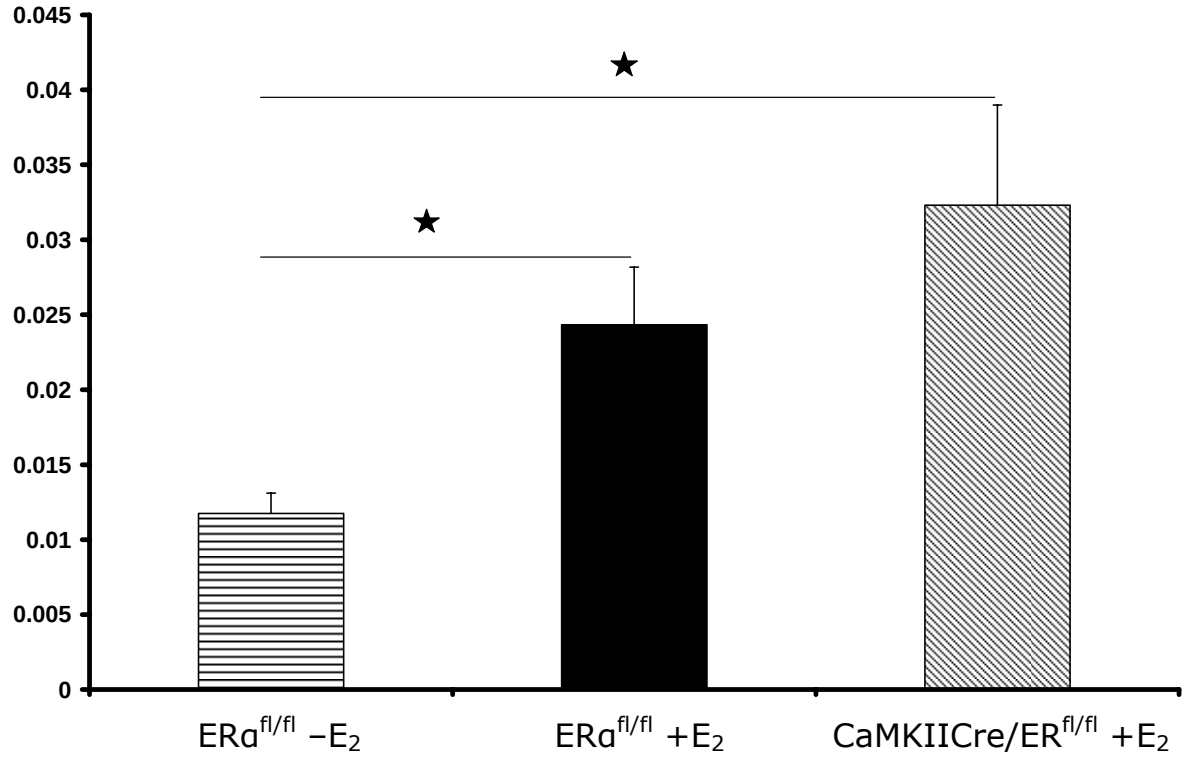

Fig. 20 Real time PCR analysis of BDNF expression in female CaMKIICre/ER$^{fl/fl}$-mice

The BDNF expression level was analyzed using real time PCR technique. RNA from the ipsilateral cortices of ERα$^{fl/fl}$-mice in absence (left bar) and presence (middle bar) of E_2 and CaMKIICre/ER$^{fl/fl}$-mice treated with E_2 (right bar) was isolated following 24 h of a MCAO.

4. Discussion

E_2 has neuroprotective effects in stroke. It was shown that physiological doses of E_2 are sufficient to reduce the stroke volume in several models of a MCAO in rodents (Dubal et al., 1998; Hurn et al., 1995; Merchenthaler et al., 2003). In contrast to these findings, the women's health initiative revealed that long term treatment with estrogens can lead to dementia and a higher risk for cardiovascular events including stroke (Schmidt et al., 2006). There are also unwanted side effects upon long term treatment with estrogens in women observed, like a higher risk for endometriosis and ovarian cancer. This controversy of "good effects" of E_2 in animal models of stroke and on the other hand "bad effects" upon long term treatment with estrogens in humans, shows clearly that there is a need for a better understanding of estrogen action in stroke.

It was demonstrated by Wise and coworkers that the neuroprotective effects of E_2 are mediated via ERα and not ERβ in mice (Dubal et al., 2001). However, since germline ER knock out mice were used in the study of Wise and coworkers, the question remained unanswered in which celltype ERα mediates its neuroprotective effects upon ligand dependent activation.

The aim of this work was to identify the celltype in which ERα action mediates neuroprotection. Therefore, three different tissue specific ERα knock out mouse strains were generated using the Cre-loxP-system. Neuronal specific ERα knock out mice were achieved by breeding $ER\alpha^{fl/fl}$-mice to CaMKIIαCre-mice (Casanova et al., 2001; Wintermantel et al., 2006). Microglial specific ERα knock out were achieved by breeding $ER\alpha^{fl/fl}$-mice to LysMCre-mice (Clausen et al., 1999). Finally, endothelial specific $CreER^{T2}$ expressing mice were analysed for endothelial specific recombination upon tamoxifen treatment, and then bred to $ER\alpha^{fl/fl}$-mice to achieve an endothelial specific ERα knock out.

Performing MCAO experiments with CaMKIICre/ER$^{fl/fl}$-mice and LysMCre/ER$^{fl/fl}$-mice revealed that the neuroprotective effects of E_2 are mediated through neuronal ERα and not microglial ERα.
After identifying neuronal ERα as the critical mediator of E_2-induced neuroprotection, female neuronal specific ERα knock mice were used to investigate the molecular mechanisms which are affected by the ERα in stroke.

4.1 In Tie2CreER^{T2}-mice, CreER^{T2} mediated endothelial specific recombination is induced upon tamoxifen treatment, but is not sufficient for complete deletion of ERα in endothelial cells of the vascular system

The Tie2-gene is expressed in endothelial cells of the vascular system and in hematopoietic cells while embryonic development, but its expression is restricted to the endothelial cells of the vascular system after birth (Takakura et al., 1998). Former generated constitutive active Tie2Cre-mice showed recombination activity of the Cre not only in endothelial cells of the vascular system but showed also recombination activity in hematopoietic cells (Constien et al., 2001) due to the activity of the Tie2 promotor while embryonic development. To circumvent Cre-mediated recombination in the hematopoietic system, transgenic mice were generated, expressing the tamoxifen inducible CreER^{T2}-recombinase under control of the Tie2-promotor.
To investigate endothelial specific recombination upon tamoxifen treatment, Tie2CreER^{T2}-mice were crossed with RAGE$^{eGFP/+}$-mice. Endothelial cells of the aorta, liver, kidney and brain of Tie2CreER^{T2}/RAGE$^{eGFP/+}$-mice were eGFP positive upon intraperitoneal tamoxifen injection. These results demonstrated that recombination is inducible by tamoxifen and that recombination is restricted to the endothelial cells of the vascular system. Tissue specific expression of the

Cre is dependent on the promotor and its regulatory sequences which drive the expression. The advantage of the use of a BAC as a vector is to include all regulatory elements of a promotor into the Cre-transgene, therefore guaranteeing the tissue specific expression. Former generated constitutive active Tie2Cre-mice used plasmids as a vector for the transgene. Since a plasmid cannot mirror the genomic surrounding of the Tie2-promotor, these mice showed recombination in the germline (Constien et al., 2001), resulting in a complete null allele. Germline recombination was not observed in the Tie2CreER^{T2}-mice analysed in this study. This finding points out the importance of the use of the whole Tie2-promotor. Experiences of this laboratory in the use of Cre-transgenes, revealed that the use of a BAC as a vector allows control of the expression of the transgene by nearly all promoter-elements of the expression driving gene. In contrast, experiences with plasmid-transgenes showed that the expression of some plasmid-based transgenes differ from the endogenous expression pattern of the genes used for the control of the transgene expression due to lacking regulatory elements. Furthermore Arnold and coworkers generated a plasmid based inducible Tie2CreER^{T2}-mice displaying difficulties in inducing tamoxifen dependent recombination (Forde et al., 2002). The authors claimed that the expression level of the transgene is not sufficient to obtain tamoxifen induced complete recombination in the endothelial cells of the whole vascular system.

The Tie2CreER^{T2}-mice investigated in this study showed recombination in the endothelial cells of all investigated organs, suggesting that tamoxifen treatment induces recombination in all vessels.

In contrast to the tamoxifen induced recombination demonstrated in the Tie2CreER^{T2}/RAGE$^{eGFP/+}$-mice of this study, the tamoxifen induced CreER^{T2}-mediated recombination was not sufficient for the complete deletion of ERα in the endothelial cells of the vascular system. Since Cre-mediated recombination is a stochastic event (Nagy, 2000), this controversy might be due to the fact that eGFP-expression is achieved by the recombination of one RAGE^{eGFP}-allele, whereas for the deletion of ERα

both alleles of the gene have to be recombined. The expression level of the $CreER^{T2}$ might be insufficient to mediate recombination of both $ERα^{fl/fl}$-alleles.

However, to reveal the reason for this controversy of complete tamoxifen induced recombination of the $RAGE^{eGFP}$-allele and incomplete recombination of the $ERα^{fl/fl}$-alleles in endothelial cells of the vascular system, further investigations have to be done.

4.2 Neuronal ERα mediates the neuroprotective effects of E_2 and not microglial ERα

To define the role of neuronal ERα in stroke, CaMKIIαCre mice were crossed with $ERα^{fl/fl}$-mice. It was demonstrated, that the resulting neuronal ERα knock out mice lacked ERα in all neurons of the cortex.

To generate a microglial ERalpha knockout, lysMCre mice were mated with $ERα^{fl/fl}$-mice, which resulted in ERα loss in the monocytic cell lineage and therefore to the loss of ERα in 92% of the microglial cells of the brain, as it was shown in this work.

Performing a MCAO experiment with female neuronal ERα knock out mice showed that the neuroprotective effect of E_2 was completely lost in the mutants. Also in the male neuronal ERα knock out mice, E_2 did not reduce the stroke volume in the mutant mice. To exclude secondary effects of the mutation, physiological parameters of the mice were monitored. There were no significante alterations in the monitored physiological measurements comparing $ERα^{fl/fl}$-mice with $CaMKIIα/ER^{fl/fl}$-mice, showing that the phenotype is due to the deletion of ERα in neurons and not because of the integration site of the Cre-transgene. These data provide evidence for the critical role of neuronal ERα to mediate the neuroprotective effects of E_2 in stoke.

It has been reported that microglial ERα plays an important role reducing damage and inflammatory events in *in vitro* models of stroke (Bruce-

Keller et al., 2000; Dimayuga et al., 2005; Ghisletti et al., 2005). However, *in vivo* models for demonstrating if there is a neuroprotective effect in stroke mediated by microglial ERα have not been investigated so far.

In contrast to the reported *in vitro* experiments, the neuroprotective effect of E_2 was still present in the micrglial ERα knock out mice after 48 h of MCAO. This shows that microglial ERα has no role in mediating E_2 dependent neuroprotection. Nevertheless, the role of microglial ERα in repressing inflammatory events and therefore promoting the regeneration of the brain after a stroke occurred, have to be investigated in long term MCAO experiments.

Taken together these experiments demonstrated that E_2 has neuroprotective effects in stroke in female as well as in male mice. Furthermore it was demonstrated, that E_2 dependent neuroprotection is mediated via neuronal ERα and not microglial ERα in the acute phase of a stroke.

4.3 Analysis of gene expression

To examine the molecular mechanisms which are affected by the neuroprotective action of ERα, real time PCR experiments were performed. Finsen and coworkers have shown that hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) expression is not altered while a MCAO (Meldgaard et al., 2006). Thus, HPRT1 was used as a reference gene for all real time PCR experiments in this study to quantify the changes in the RNA-expression levels of the investigated genes.

4.3.1 ERα is upregulated upon a MCAO

Since immunohistochemistry for ERα showed that the receptor is expressed at low levels in the cortex, a real time PCR experiment was performed to analyse ERα expression in stroke. Comparing the ipsilateral cortex which is affected by the MCAO to the unaffected contralateral cortex, showed significant upregulation of ERα upon MCAO in E_2 treated as well as in untreated mice. These findings give another hint to the important role of neuronal ERα in stroke.

4.3.2 COX-2 expression is only elevated in neuronal specific ERα knock out mice, but does not respond to E_2

COX-2 plays different roles in the mammalian brain. While the perinatal phase COX-2 and one of its products prostaglandin E2 are needed for the development of the neuronal structures which mediate sexual behaviour (Amateau and McCarthy, 2004). Additionally the expression level of COX-2 is affected by E_2 while this perinatal phase.
On the other hand, COX-2 is also known to have neurodegenerative effects in stroke (Hara et al., 1998), mediated by increased PGE2 production and binding of PGE2 to its receptor EP1 (Kawano et al., 2006).
To elucidate the controversy that E_2 on the one hand has neuroprotective effects but on the other hand is able to induce COX-2 expression, which is described to exacerbate neuronal death in ischemia, COX-2 expression was investigated by real time PCR. COX-2 expression was not significantly changed in ERα$^{fl/fl}$-mice treated with E_2 compared to ERα$^{fl/fl}$-mice without E_2 after 24 h of MCAO. In contrast, neuronal ERα knock out mice showed a 27% elevation of COX-2 expression compared to E_2 treated ERα$^{fl/fl}$-mice. E_2 alone had no effect on the level of COX-2 expression, only the loss of the whole receptor in neurons in the CaMKIIα/ER$^{fl/fl}$-mice leads to an increased expression of COX-2. This data provide a hint that COX-2

expression is not influenced by E_2, but might be repressed by ERα. However, expression-levels of downstream signalling molecules of COX-2, like EP1 and EP2, were not changed in MCAO, leading to the conclusion, that COX-2 plays no pivotal role in E_2- and ERα-mediated neuroprotection in ischemia.

4.3.3 BDNF is upregulated upon E_2 treatment, but its regulation is independent from ERα

There are several effects of BDNF in the brain, like trophic functions while development and neuroprotective actions in models of brain injury and stroke (Behl, 2002; Garcia-Segura et al., 2001). These mechanisms are described to be influenced by E_2. Like demonstrated here, BDNF is upregulated in stroke upon E_2 treatment. But this upregulation is independent of neuronal ERα, since the BDNF expression level is also elevated in the neuronal ERα knock out mice compared to the $ER\alpha^{fl/fl}$-mice lacking E_2 treatment. However, the neuroprotective effects of BDNF seem to be upstream of ERα action in stroke. BDNF is upregulated in stroke upon E_2 treatment independent of neuronal ERα, but the neuronal damage can be only prevented in the presence of ERα in neurons since the stroke volume and therefore the neuronal damage is only reduced in the E_2 treated $ER\alpha^{fl/fl}$-mice. These data demonstrate, that BDNF is necessary for neuroprotection in stroke, but it is not sufficient. Furthermore it was shown that neuronal ERα is required to mediate the neuroprotective effects of BDNF in a MCAO. The upregulation of BDNF is E_2 dependent, whether this upregulation is dependent on ERα in astrocytes or ERα in other celltypes of the brain except neurons could not be answered in this work.

4.4 Future perspectives

It was demonstrated in this work, that E_2 has neuroprotective effects in female as well as in male mice in a model of a MCAO. Furthermore it was shown, that the neuroprotective effects of E_2 are mediated via neuronal ERα and not microglial ERα using the Cre-loxP system to generate tissue specific ERα knock outs. ERα is upregulated upon stroke, pointing to its important role in neuroprotection. Furthermore, neuronal ERα is needed to mediate the neuroprotective effects of BDNF in stroke. However, downstream signalling molecules of neuronal ERα mediated neuroprotection have not been identified. Therefore microarray analysis of RNA isolated from the cortices of neuronal specific ERα knock outs should reveal possible downstream signalling molecules of neuroprotective ERα action. Validated E_2 targets can be used to "rescue" the phenotype of CaMKIIα/$ER^{fl/fl}$-mice in a model of a MCAO, and therefore give new insights in molecular mechanisms mediating neuroprotective actions in stroke and potentially give rise to new medical applications.

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